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CONTENTS OF VOLUME 38

No. 1, March, 1951

PAGE
YOSHIKAWA Haruhisa. The oxidation-reduction potential of cytochrome b′, a hemochromogen-like component of cytochrome in liver 1
ARAYA Shimpei and KOBAYASHI Mosaburo. Protein denaturation and biuret reaction 6
ANAN Koichi. The peroxidatic activity of catalase 19
OHTA Kiyohiko. Studies on nucleic acid metabolism. XIX. A synthesis of 2-desoxy-D-ribose 31
ISHI Tôru. Action of alloxan on adrenalectomized rabbits 37
KAWAGOE Hisao. Some studies on the group specific substance in human saliva 45
MIURA Yoshiaki. On the tuberculostatic action of various organic compounds. III. Benzene, pyridine, and thiazole derivatives 69
OGURA Yasuyuki. Studies on glucose dehydrogenase of *Aspergillus oryzae* 75
EGUCHI Satsuki. Amino acids of *Mycobacterium tuberculosis* 85
SAKAGUCHI Shoyo. A note to the colorimetric determination of arginine 91
ABSTRACTS from The Journal of Japanese Biochemical Society (*Seikagaku*).
    Volume 20 (1948) I
    Volume 21 (1949) III

No. 2, June, 1951

YAMASAKI Kazumi. Isolierung der Tetraoxynorsterocholansäure aus der Hühnergalle und über die Gallensäuren der Galle von *Citellus mongolicus ramosus*, Thomas "Hataris" und von Schafen 93
SHIMIZU Taiji. Pyruvate and α-ketoglutarate in blood and urine. I. Normal values and the effects of exercies and thiamine or riboflavin injection 99
NIWA Koyata. Purification of β-glucosidase of *Aspergillus niger*. II. 109
KURODA Yoshio. Studies on protamine. III. 115
MARUYAMA Mitsunori. Chemical aspect upon the water complement and the so-called fourth component of the complement. 119
HIROHATA Ryozo, YAMASAKI Seiichiro, ISEKI Toshinori, TO Tokuwa, ISEDA Syun, and SHOJIMA Masataka. Studies on ω-oxidation. I. Fate of L-rhodinic acid and its allied acids in organism 135
YAMAMOTO Toshiichi. Studies on lipase. I. On the activation of pancreas lipase 147
NAKAYAMA Teishi. Studies on the conjugated lipids. II. On cerebron sulphuric acid 157
YAGI Kunio. Microdetermination of riboflavin, flavin mononucleotide and flavin adenine denucleotide by filter paper chromatography 161
SEKINE Takamitsu. Cholinesterase in pig spermatozoa 171

SIBATANI Atsuhiro and FUKUDA Michio. Importance of controlling water content of the solvents for paper chromatography 181
YAMAKAWA Tamio and SUZUKI Shizue. The chemistry of the lipids of post-hemolytic residue or stroma of erythrocyte. I. Concerning the ether-insoluble lipids of lyophilized horse blood stroma 199
ABSTRACTS from The Journal of Japanese Biochemical Society (*Seikagaku*) Volume 22 (1950) .... I–VIII

No. 3, September, 1951

KAZIRO Koozoo and Goro KIKUCHI. Studies on the interaction between pyridine-hemin and hydrogen peroxide or oxygen. I. Spectroscopic observations on the process of verdohemochrome formation with special reference to its quantitative treatment 213
SHUKUYA Ryoiti. On the kinetics of the human blood cholinesterase 225
SUZUOKI-Ziro and SUZUOKI-Tuneko. Carbohydrate metabolism of *Trichomonas foetus* 237
MORITA Sigehiro, SUZUKI Koichi and TAKASHIMA Shiro. On the cause of the S-shaped rate-light intensity relationship in the photosynthesis of purple bacyeria 255
FUJITA Akiji and AOYAMA Masataro. Colorimetric determination of vitamin A with glycerol dichlorohydrin 263
FUJITA Akiji and AOYAMA Masataro. Flurorometric determination of vitamin A 271
YAMAMOTO Toshiichi. Studies on lipase. II. On the histidine activation of pancreas lipase 277
SUDA Masami, HASHIMOTO Kizo, MATSUOKA Hiroshi and KAMAHORA Takeshi. Further studies on pyrocatecase 289
SUDA Masami, TAKEDA Yoshiro, SUJISHI Kiyoshi and TANAKA Takehiko. Metabolism of tyrosine. III. Relation between homogentisicase, ferrous ion, and L-ascorbic acid in experimental alcaptonuria of guinea pig 297
OGATA Tomio, SHIMANOUCHI Takehiko and YAMAOKA Seizaburo. Histamin contraction of a guinea pig smooth muscle at lower temperatures with reference to adsorption energy 303
ABSTRACTS from The Journal of Japanese Biochemical Society (*Seikagaku*) Volume 22 (1950) .... IX–XV

No. 4, December, 1951

MURATA Kiku and UEBA Akiko. On the specificity of aneurinase. II.......... 309
KOSAKI Takekazu. On the pathogenesis of porhyria, especially of the toxic porphyria after the administration of sulfonal derivatives 317
TAKEMOTO Nobuaki. On the metabolism of chloic acid in organism............ 325
EGAMI Fujio and ITAHASHI Michiko. Enzymatic decomposition of nitromethane by liver homoganates 329

YAMAMOTO Toshiichi. Studies on lipase. III. On the activation of liver esterase 335
YONEYA Toshio. Studies on lysine and its derivatives. I. The asymmetric anilide synthesis of lysine derivatives by the action of ficin 343
AKAMATSU Shigeru and SEKINE Tsutomu. Hydrolysis of arginine by *Stroptococcuscus faealis* 349
AKAMATSU Shigeru and KOBAYASHI Gaijiro. Studies on the specificity of heteroarginase 355
FURIHATA Rikio. Studies on the agglutinating and hemolysing factors contained in *Ricinus communis*. I. Studies on the agglutinating factor 361
ABSTRACTS from The Journal of Japanese Biochemical Society (*Seikagaku*) Volume 23 (1951) .... XVI–XXIII

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The Journal of Biochemistry Vol. 38, No. 1.

THE OXIDATION-REDUCTION POTENTIAL OF CYTOCHROME b', A HEMOCHROMOGEN-LIKE COMPONENT OF CYTOCHROME IN LIVER

By HARUHISA YOSHIKAWA

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Received for publication, September 11, 1950)

The typical spectrum of reduced cytochrome described by Keilin (*1*), possesses four bands, a (604 mμ), b (564 mμ), c (550 mμ) and d (520 mμ). Thoracic muscle of bee, baker's yeast and mammalian heart muscle show this type of cytochrome spectrum, which I shall call heart type. In numbers of organisms, another type of spectrum has been seen, which shows hemochromogen-like bands lying at 560 mμ and 525 mμ. Tissues of some invertebrates, bottom yeast and some bacteria (for instance *E. colli* (*2, 3*)) belong to this group. This modified spectrum has been observed in mammalian liver cells (*4*) as well as in succinodehydrogenase preparation from pig kidney (*5*). I shall call the hemochromogene-like component cytochrome b' according to Fujita and Kodama (*3*), and this type of spectrum liver type.

Although the characteristics of cytochrome with heart type spectrum have been studied in detail, those of cytochrome with liver type spectrum have celled little attention. In this paper some observations on cytochrome b' will be presented.

EXPERIMENTAL

Crude oxidase preparation was prepared according to Keilin and Hartree (*5*). For the spectroscopic examination, two instruments have been used—the handspectroscope of Zeiss and the wavelength spectrometer of Fuess.

RESULTS

Spectroscopic Observations on Various Organs of dog, rabbit and rat were perfused with saline solution to wash out the blood, and examined with the spectroscope.

TABLE I

Components of Cytochrome Present in Various Tissue Preparations

Animal	Preparation of organs	Cytochrome			
Rabbit	Heart (slice)	a	b		c
〃	Liver (slice and enzyme prepn.)			b′	
〃	Kidney (slice and enzyme prepn.)	a		b′	
〃	Ovary			b′	
〃	Brain (homogenate)	a	b		c
〃	Testis (homogenate)		b		c
Rat	Liver (enzyme prepn.)			b′	c
〃	Kidney (enzyme prepn.)	a		b′	
Dog	Heart (enzyme prepn.)	a	b		c
〃	Liver (〃)			b′	
〃	Kidney (〃)	a		b′	
〃	Heart (〃)	a	b		c

The results are presented in Table I. Liver and ovary show the hemochromogene-like bands of b′ instead of typical four bands of cytochrome. In rat liver, c band was seen at 550 mμ superposed on more diffuse absorption band of b′. Although no c band was detected in liver tissue of rabbit and dog by direct spectroscopy, a solution with faint absorption band of cytochrome c, was obtained by the method of Keilin and Hartree (*6*) for preparation of cytochrome c, indicating the presence of cytochrome c in liver cells.

Kidney tissue showed a band as well as b′.

Behavior of Cytochrome b′ in Oxidation and Reduction—It is readily seen from Table II that, like b component, b′ component is autoxidizable in air, even when the cytochrome oxidase is poisoned by cyanide. The fact that hydraquinone does not reduce b′ component, suggests that the oxidation-reduction potential is much lower than that of a and c, which can be reduced by hydroquinone.

The Oxidation-Reduction Potential of Cytochrome b′—In order to estimate the oxidation-reduction potential of cytochrome b′ the following experiment was carried out.

2 ml. of the enzyme preparation (pH 7.5) obtained from dog liver were received in a test tube, and 2 ml. of oxidation-reduction dye solu-

TABLE II

Behavior or Cytochrome Components in Oxidation and Reduction

Preparation	Treatment	Absorption bands			
Rat liver enzyme preparation	Reduction by hydroquinone				c
	Reduction by hydrosulfite			b′	c
Dog kidney enzyme preparation	Reduction by hydroquinone	a			
	Reduction by hydrosulfite	a		b′	
	〃 + NaCN	a		b′	
	〃 + NaCN, aeration	a			
Ox heart enzyme preparation	Reduction by hydroquinone	a			c
	Reduction by hydrosulfite	a	b		c
	〃 + NaCN	a	b		c
	〃 + NaCN, aeration	a			c

TABLE III

Estimation of the Redox Patential Cytochrome b′ by using of Oxidation-Reduction Indicators

+: Appearance of cytochrome b′ band. ++: Full development of b′ band. Figures in brackets represent potentials of mixtures. (Temperature 15°, pH 7.5).

Oxidation-reduction indicator	% Reduction of dye			
	25%	50%	75%	90%
K-indigotetrasulfonate	− (−0.05)	− (−0.07)	− (−0.08)	− (−0.10)
K-indigotrisulfonate	− (−0.09)	− (−0.10)	− (−0.12)	+ (−0.13)
Nile blue	+ (−0.13)	++ (−0.14)	++ (−0.16)	++ (−0.17)

tion were added. Potassium indigotetrasulfonate $E_0'=-0.07$ volt), potassium indigotrisulfonate ($E_0'=-0.10$ volt) and nile blue ($E_0'=-0.14$ volt) were used as indicator dyes.

The mixture, after it was covered with liquid paraffin, was added with a diluted sodium hydrosulfite solution drop by drop, until the absorption band of reduced cytochrome b′ appeared. At this point, the color intensity of the dye in the mixture was compared with the standard mixture, which had been prepared by mixing equal volumes of the enzyme preparation and appropriately diluted dye solution, containing 25, 50, 75 or 90 per cent of the original solution.

The results are given in Table III. It is readily seen from the table that the oxidation-reduction potential is approximately −0.13 volt at pH 7.5.

Action of Pyridine on Enzyme Preparation—When pyridine and sodium hydrosulfite were added to enzyme preparation made from dog liver, absorption band of cytochrome b′ became narrower and stronger. This suggests that cytochrome b′ converted to pyridine-hemochromogene.

DISCUSSION

Ball (*7*) reported the oxidation-reduction potential of b component of cytochrome to be—0.04 volt at pH 7.4. Much lower potential, −0.13 volt, was obtained in the case of cytochrome b′ in the liver preparation. Such a low potential is comparable with potentials of hemochromogenes composed of protoheme and imidazole compounds, such as histidine (−0.11 volt), pilocarpin (−0.13 volt) and globin (−0.98 volt at pH 7.06), determined by Barron (*8*).

Yakushiji and Mori (*9*) reported that cytochrome b isolated from yeast cells was converted to pyridine-hemochromogene, when pyridine and reducing agent were added. They observed two absorption spectrum bands at 564 mμ and 532 mμ after reduction of isolated cytochrome b with hydrosulfite. They also described that protohematin combined with flavoprotein developed absorption bands very similar to those of cytochrome b after reduction, while pyridine-hemochromogene-like but more diffuse bands were seen with albumin of denatured yeast protein. It is quite possible that the position of bands as well as the oxidation-reduction potential of hemochromogene may very with the nature of nitrogen compound attached to the heme.

It is well known that the absorption spectrum of cytochrome in yeast may be either heart type or liver type, depending on the mode of cultivation. If the yeast is cultivated anaerobically, the four banded cytochrome is converted to hemochromogene-like substance. If it is cultivated aerobically, the reverse change take place (*10*, *11*).

These experimental evidences suggest that heme in cell exists as a protein-hemochromogene which prossesses the most suitable potential for cellular function under given condition, the lower potential of cytochrome b′ being suited for respiratory process under lower oxygen tension. It is to be recalled here that liver is the unique organ in that it is supplied oxygen chiefly by venous blood from portal vein.

SUMMARY

1. Hemochromogene-like component of cytochrome, cytochrome b′, is autoxidizable like as cytochrome b.
2. The level of oxidation-reduction potential of cytochrome b′ is approximately −0.13 volt.
3. The meaning of such lower potential of cytochrome b′ than that of cytochrome b has ceen discussed.

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PROTEIN DENATURATION AND BIURET REACTION

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Received for publication, Sept. 11, 1950)

INTRODUCTION (*1*, *2*)

The denaturation of protein is essentially a modification of the cofiguration of native protein where the peptide chain composing the protein is thought to unfold. (*3*, *4*) Thus, as changes take place in the configuration of protein by denaturation, very marked effects are seen in solubility, viscosity, *etc.* Chemically, also, the action of the various groups in the protein is known to increase, especially the increase of the activity of the SH and S-S groups, which were quantitatively examined by Mirsky, Anson, *et al.* (*5*, *6*, *7*) gives us new grounds to consider the problem of protein denaturation.

The configuration of native protein is maintained by various secondary linkages which fasten the peptide chain, and particularly among such secondary linkages the linkage between the NH in a peptide linkage part and the CO of another peptide linkage part may be important. Mirsky and Pauling (*8*), *et al.* considered the hydrogen bond to be the linkage between them.

Therefore, when protein is denatured, at least a part of the linkage of this kind will be broken and the peptide linkage part will change into a more free form. As a result the activity of the peptide linkage part should naturally increase. Although this has been already assumed by the enzyme action (*9*, *10*, *11*), it is not conclusive. Therefore, in order to directly prove this fact one of the authors, S. Araya (*12*), applied the biuret reaction as the reaction for the peptide linkage part. But by the ordinary biuret reaction using NaOH he could not find any difference between the native and the denatured ovalbumin. Therefore, thinking that this result may be due to the fact that the native ovalbumin will also be denatured by NaOH in the biuret reaction, S. Araya modified the biuret reaction by using a diluted solution of Na_2CO_3, a weak alkali, intead of NaOH. By this modification one can find a distinct difference between the native and denatured protein.

With the denatured ovalbumin violet-purple color, which is the characteristic color in the biuret reaction, appears immediately after the reaction, while with the native ovalbumin the violet-purple color does not appear immediately but begins to appear gradually only after several minutes.

The authors measured quantitatively this weak alkali biuret reaction by the Pulfrich-Stufenphotometer and studied the reactivity of the peptide linkage par, using various method of denaturing and various kinds of proteins. As a result, the authors found that this method shows the denatured state of protein very accurately.

PREPARATION OF THE PROTEIN SAMPLES

The proteins used in the experiment were ovalbumin, horse, serum albumin, euglobulin, pseudoglobulin and albumin from egg (Takeda). The method of preparation of eace is as follows:

Ovalbumin.—This was prepared by Keckwick's (*13*) Na_2SO_4 method using the hen's egg. The ovalbumin was recrystallized 3 times, and the crystals were filtered and dried at room temperature by using an electric fan. The ovalbumin crystals are dissolved in distilled water and dialyzed by using a cellophane bag. After being dialyzed for several days the solution is filtered through a filter paper (Toyo Filter Paper Company's No. 6), diluted to the proper density and used (the N in the filtrate measured by the Micro-Kjeldahl method, and all the protein solutions were used immediately after being prepared).

Horse Serum Albumin—The serum albumin was crystallized by Keckwick's method (*14*), filtered, dissolved in distilled water, the amount of which being about 1/5 of that of the serum, and extracted for about 10 hours at 30°. This solution is filtered; Na_2SO_4 and $N/5$ H_2SO_4 are added to the filtrate and recrystallized. The same recrystallization process is repeated two more times. Then, the same procedure is used as in the case of ovalbumin.

Horse Serum Globulin—By Howe's method (*15, 16, 17*) Na_2SO_4 powder is added to the horse serum in the amoumts of 10.6, 13.6, 17.5 and 21.5 g./dl, and fibrinogen, euglobulin, pseudoglobulins I and II are isolated. The sediments of each fraction are dissolved in about 1/5 serum amount of distilled water and filtered. To the filtrate is added Na_2SO_4 in amounts necessary for fractionation. This procedure is repeated again, and then the procedure explained in the case of ovalbumin is used.

Albumin Mode from Egg (*Takeda*)—A certain amount of albumin made from egg (Takeda) is dissolved in distilled water, dialyzed by use of cellophane bag, adjusted to pH 4.6, filtered, the N measured and used according to the procedure explained as for the other albumins.

EXPERIMENTAL METHOD

In the experiments using the Pulfrich-Stufenphotometer, with certain exceptions, 0.2 % protein solution is used. To 3.0 ml. of this protein solution, about 0.3 ml. of 1 % Na_2CO_3 and 0.13 ml. of 1 % $CuSO_4 \cdot 5H_2O$ are added and the biuret reaction is done with pH 9.2-9.4. The resulting violet-purple color is measured by the Pulfrich-Stufenphotometer using a cuvette with a thickness of 0.99 cm. The pH of the reacting solution is adjusted accurately by regulating the amount of Na_2CO_3, but the amount of $CuSO_4$ is kept constant. All the pH value is measured by the pH test paper (Toyo Filter Paper Co.).

For the purpose of selecting a filter for the photometer, the extinctions are measured by using different kinds of filters I hour after the ordinary biuret reaction of the protein solution. The maxinum of the extinction appeared around 550 mμ, so the S_{57} filter is used in all cases.

RESULTS

Hdat Denaturation—(a) Fig. 1 shows the comparison of a 0.5 % albumin, made from egg (Takeda), denatured by being put into a water bath at 80° for 10 minutes, and the native albumin of the same kind. The ordinate represents the extinction and the abscissa the time after the buiret reaction. As shown in the figure, in the case of (A), (B), and (C) the amount of Na_2CO_3 added is gradually increased and according to the amount added the coloring becomes gradually rapid. (D) is the case of the ordinary method of biuret reaction using NaOH. In this case the curves of both the denatured and native are the same and overlap each other from the beginning. As the result shows, in comparing the denatured protein with the native by this method, the pH of both solutions in the biuret reaction should be kept exactly at the same value. The pH of (A) is 9.0, so all the following experiments are carried out with the pH around 9, the exact value being shown in each figure.

(b) The 0.2 % protein solutions of several kinds were heated in

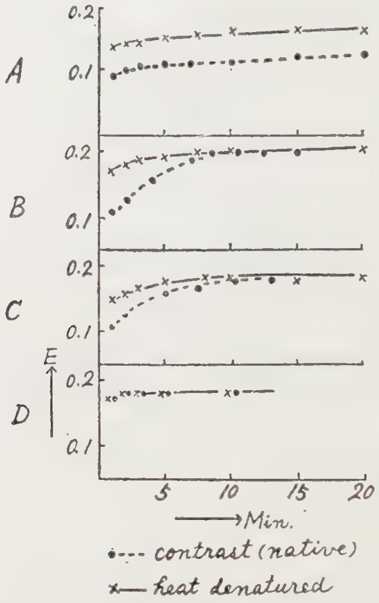

FIG. 1. Influence of heat denaturation

A.	0.5% Ovalbumin	5 ml. +	1% Na_2CO_3	0.4 ml. +	1% $CuSO_4$-$5H_2O$	0.25 ml.
B.	,,	3 ml. +	,,	1 ml. +	,,	0.15 ml.
C.	,,	3 ml. +	,,	2 ml. +	,,	0.15 ml.
D.	,,	3 ml. +	10 %NaOH	2 ml. +	,,	0.15 ml.

boiling water for 5 minutes and cooled in cold water, and the weak alkali biuret reaction of these solutions was attempted together with the native protein solution as contrast. The results are shown in Fig. 2. The dotted line represents the native protein while the solid line represents the heat denatured protein. In all the cases the biuret reaction occurs much more actively in the case of the heat denatured protein than in the case of the native. Furthermore, even in the native proteins it is seen that the reactivity of the peptide linkage part is also appreciably different between these kinds of proteins.

Next, in a similar way the biuret reaction of 0.5 % Witte's peptone and 0.5 % casein, which is difficult to be denatured by heating, were observed. As shown in Fig. 3, there is absolutely no difference between the heat treated ones and the ones without any treatment.

Urea Denaturation—As described in the introduction, when most of

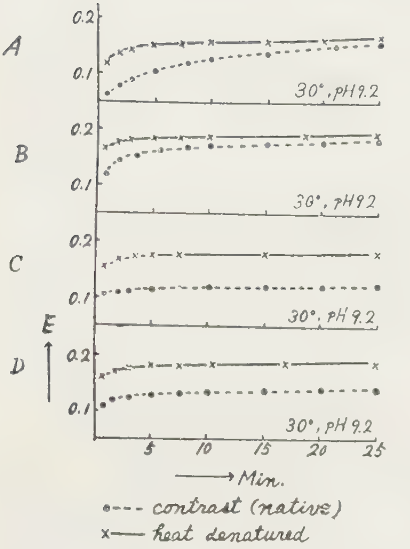

FIG. 2. Influence of heat denaturation

A. Ovalbumin
B. Serum albumin
C. Euglobulin
D. Pseudoglobulin I

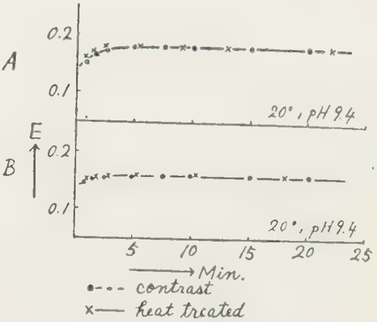

FIG. 3. Influence of heat treatment

A. Witte's peptone
B. Casein

the protein is denatured, the SH or S-S groups in the protein become more reactive. The work by Mirsky and Anson, *et al.* who investigated quantitatively these facts is valuable for the recent study on protein denaturation. Wanting to compare the activation of the SH group with the activation of the peptide linkage part, the authors carried out the following experiment:

A 1% ovalbumin is denatured by adding urea to it and the nitroprusside reaction for the SH group is attempted. The reaction becomes positive when 8 millimols of urea are added to every 1 ml. of albumin solution, but it becomes negative when the amount of urea is 7 millimols or below. This result corresponds to the results obtained by Greenstein (*18*). However, the biuret reaction in a 0.2% ovalbumin solution, as shown in (A) of Fig. 4, is activated by adding 1 millimol of urea to every 1 ml. of albumin solution, and, furthermore, as the urea concentration is increased the reaction becomes further activated. And in studying the biuret reaction of euglobulin and pseudoglobulin I, as

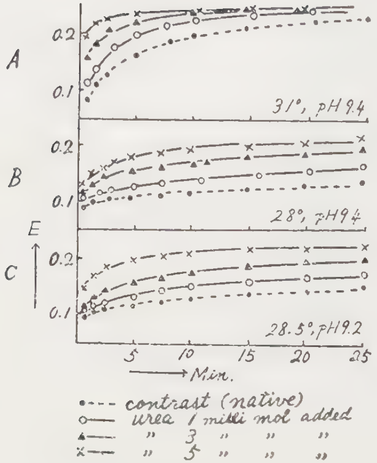

FIG. 4. Influence of urea.

A. Ovalbulin
B. Euglobulin
C. Pseudoglobulin

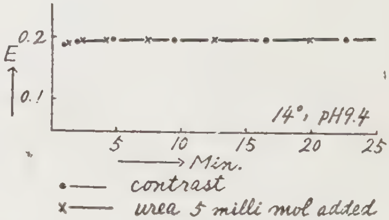

FIG. 5. Influence os urea
(Witte's peptone)

shown in (B) and (C) of Fig. 4, the activation of the reaction was observed even when 1 millimol of urea was added to 1 ml. of protein solution. In these cases the urea itself naturally is indifferent to the biuret reaction. For example, in the biuret reaction of 2% Witte's peptone solution, as shown in Fig. 5, the curves of both the peptone solutions with urea 5 millimols per ml. and without urea overlap each other.

From these facts it seems that the influence of denaturation appears more delicately on the peptide linkage part than on the SH group.

Ultraviolet Denaturation—Into the Petri's dish measuring 10 cm. in diameter, 20 ml. of protein solution (0.2%) are poured and the dish is exposed to therapeutic sun lamp rays at a distance of 30 cm. During the exposure the contents of the dish are moved lightly every few minutes. The rise in the temperature of the solution due to the exposure is 38°. As the rise in temperature of this extent has no influence, the contrast used was that left at room temperature (30°). The experiment was performed 1 hour to 3 hours after leaving at room temperature after the exposure.

As in Fig. 6, in the case of ovalbumin the biuret reaction was activated when exposed for 15 minutes and activated further when exposed for

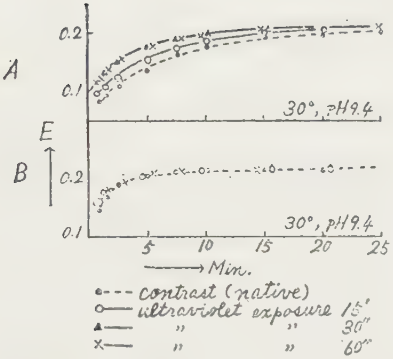

Fig. 6. Influence of ultraviolet

A. Ovalbumin

B. Serum albumin

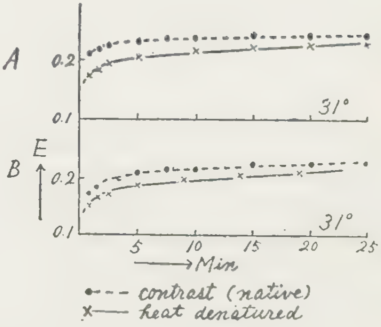

Fig. 7. Influence of formaldehyde

A. Ovalbumin

B. Serum albumin

30 minutes. When the time of exposure was 60 minutes the reactivity was the same as when the time of exposure was 30 minutes. In the case of serum albumin the influence of the ultraviolet ray was observed not so clearly as in the case of ovalbumin. This may be due to the fact that the serum albumin does not become denatured under these conditions or, as will be described in a later report, as the serum albumin is one with a strong reversal denaturation, even though the serum albumin was denatured to a certain degree, it may have immediately returned to its former native state.

The Influence of Formaldehyde—To 3 ml. of denatured protein solution (0.2% ovalbumin and serum albumin), which are heated for 5 minutes in boiling water and then cooled, and to 3 ml. of native protein solution 1 ml. of formalin is added first and a few minutes later 1.0 ml. of 10% NaOH and 0.15 ml. of 1% $CuSO_4 \cdot 5H_2O$ are added. The results, as shown in Fig. 7, is opposite to the cases without the addition of formalin, and the reactivity to the biuret reaction of the denatured protein is lowered than in the case of the native.

It seems that this fact is explained by the following assumption: When the protein is denatured, the peptide linkage part is in a more free state and then the free peptide linkage part may combine easily with the added formaldehyde. Therefore, the biuret reaction is inhibited by the formaldehyde already combined. Contrary to this, with the native protein the peptide linkage parts are mutually closed and even if the formaldehyde is added they do not combine so easily as in the case with the denatured protein. Therefore, in the case of native protein the influence of formaldehyde on the biuret reaction is smaller.

DISCUSSION

As it is clear from the above experiments, under a certain condition a distinct difference is observed between the native protein and the denatured protein by the weak alkali biuret reaction using Na_2CO_3, and as shown in the experiment of Fig. 4 this reaction is very delicate.

However, as the intensity of this biuret reaction is believed to be influenced by various conditions, it is necessary to keep the constant conditions. When the reactivity of various proteins is compared, the conditions we must consider first are pH, temperature and the concentration of Cu^{++}. It can be seen clearly that pH influences the weak alkali biuret reaction greatly from Fig. 1. Furthermore, in this case the kind of alkali used is not indifferent to the result even if the pH is kept constant. For example, as in Fig. 8, when 0.2 ml of 3 % $CuSO_4$ is added to a 0.4% ovalbumin solution (Takeda) and the pH is adjusted to 9.6 with 3% Na_2CO_3 and $N/5$ NaOH respectively, the biuret reaction of the native and heat denatured ovalbumin shows that the reactivity is more intensive with the NaOH than with the Na_2CO_3, and, furthermore, the difference between the native and the heat denatured is not so marked. But in this case when peptone (0.5%) is used instead of protein there is no difference between Na_2CO_3 and NaOH. (Fig. 9) Therefore, it seems that this phenomenon also results to the specific configuration of the protein.

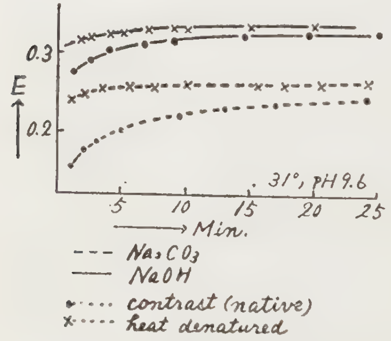

Fig. 8. Difference of NaOH and Na_2CO_3 as alkali (Ovalbumin)

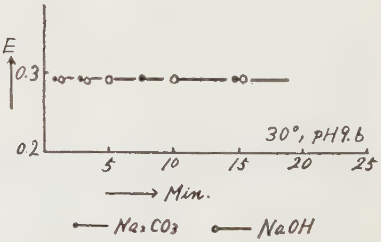

Fig. 9. Difference of NaOH and Na_2CO_3 as alkali (Witte's peptone)

As predicted, the temperature influences the weak alkali biuret reaction, examples being shown in Fig. 10 (0.4% ovalbumin (Takeda)). From this experiment the Klett-Summerson's photoelectric colorimeter with a green filter is used.)

In regard to the concentration of Cu^{++}, naturally a sufficient reaction will not take place if it is not above a certain concentration. Therefore, in this case the concentration of Cu^{++} should have a certain relationt to the amount of protein or the amount of reactive peptide linkage.

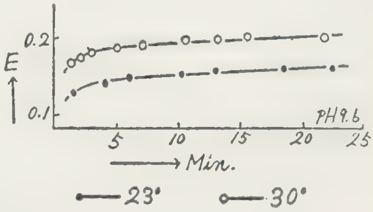

FIG. 10. Influence of temperature (Ovalbumin)

Taking into consideration the various points mentioned above, when the intensity of the weak alkali biuret reaction of the diluted horse serum of various concentration was studied, a fairly quantitative relation was recognized as shown in Fig. 11. Fig. 11 represents the intensity of the

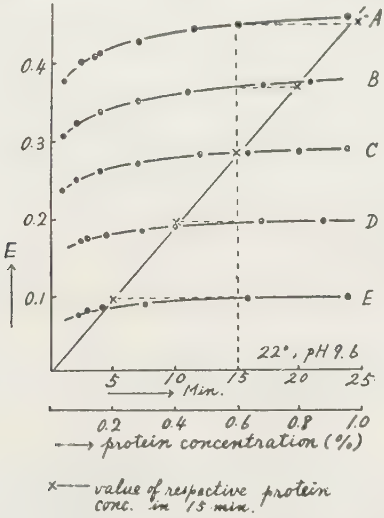

FIG. 11. Biuret reaction intensity of various concentration of serum

A. Horse serum protein 1.0 %
B. ,, ,, ,, 0.8 %
C. ,, “ ,, 0.6 %
D. ,, ,, ,, 0.4 %
E. ,, ,, ,, 0.2 %

biuret reaction of various concentrations of serum to each of which 0.3 ml. of $CuSO_4$ and about 0.3 ml. of Na_2CO_3 in corresponding concentration to that of the serum, are added and the pH adjusted to 9.6. There is a straight-line relation between the intensity of the biuret reaction after certain lapse of time and the concentration of the protein.

However, this weak alkali biuret reaction is influenced further by the various substances in the solution. The first of these is due to the substances as tartarates and citrates which are thought to easily from complex compounds with Cu^{++}. In this case the biuret reaction is markedly impeded, but as this kind of an influence can also be observed in the case of peptone, it is not peculiar to protein. (Fig. 12). The impeding action due to ammonia, uric acid, phenol, *etc.* are believed to be of the similar kind. Woreover, the inpeding action is seen also due to the sugar group as glucose, but it is assumed that the mechanism is quite complicated.

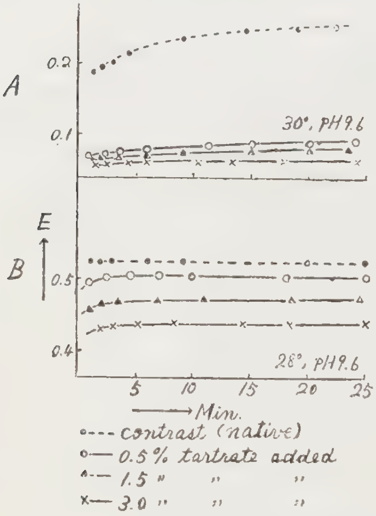

FIG. 12. Influence of tartrate
A. Ovalbumin
B. Witte's peptone

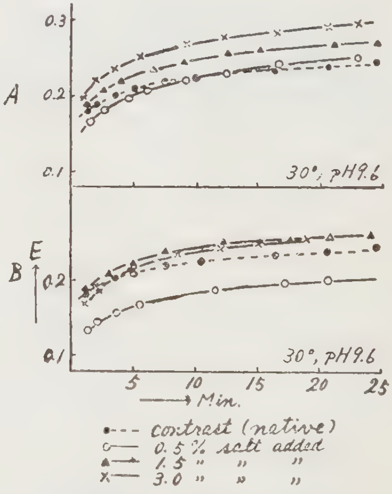

FIG. 13. Influence of salt
(Ovalbumin)
A. NaCl
B. Na_2SO_4

There is also an influence due to the ordinary neutral salts. For example, as in Fig. 13, it is influenced by NaCl and Na_2SO_4. The presence of a certain amount of salt raises the reactivity of the weak alkali biuret reaction of protein. However, when peptone (0.5%) is used instead of protein there is no such influence, as in the example in Fig. 14. Therefore, it seems that this phenomenon is due to something peculiar to protein.

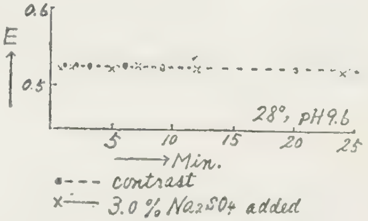

Fig. 14. Influence of salt (Witte's peptone)

As explained above, this weak alkali biuret reaction is influenced very delicately by various factors. In other words, this means that this reaction represents very delicately the configuration of the protein. Therefore, if these points are considered and if this method is carefully used, we believe that a new observation regarding the fine configuration of the protein, which herefore could not be obtained by other reactions can be obtained. It is regretful that the structure, to which the characteristic color of the biuret reaction of the protein is due, is not yet known concretely. However, deducing from the study of the biuret reaction on the simple substance and considering our experimental results of Fig. 7, at least, it is certain that this reaction is related to the peptide linkage part of the protein. Therefore, as in the experiments above so far, under a certain condition, it can be considered that this reaction represents the reactivity of the peptide linkage part of the protein and that this reactivity increases by denaturation.

SUMMARY

The following results were obtained by modifying the biuret reaction to a weak alkali.

(1) The reactivity of the peptide linkage part of various protein (ovalbumin, serum albumin, serum globulin) is clearly increased due to denaturation by heat, urea, *etc.*

(2) But when the biuret reaction is examined after the protein is acted upon by formaldehyde, the results is opposite to the case without the addition of formoldehyde, and the reactivity of denatured protein is lower than the native protein.

(3) The influence of denaturation is seen much more delicately on the peptide linkage part than on the SH group. Therefore, this Weak alkali biuret reaction represents the configuration of protein very delicately. However, as this reaction is influenced by many secondary

factors, this point must be considered very carefully.

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THE PEROXIDATIC ACTIVITY OF CATALASE

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Keilin and Hartree (*1*) reported in 1936 on the coupled oxidation of alcohol by catalase in the presence of dehydrogenase- or oxidase-system, the main feature of which was the primary formation of hydrogen peroxide, and the secondary oxidation of alcohol by catalase, where catalase played the role of the peroxidase.

Later Keilin and Hartree (*2*) reported that catalase could oxidize alcohol even with hydrogen peroxide solution, and further that the concentration of catalase required for the coupled oxidation of alcohol was about 1000 times greater than that was required for the decomposition of the hydrogen peroxide formed in the primary reaction.

Chance (*3-10*) recently reported the studies on catalase activities by his "rapid flow method": Alkyl hydrogen peroxides were combined with all three or four hematin-iron groups of catalase molecule, forming primarily the greenish complex and secondarily the red complex, analogous to the horse-radish peroxidase-alkyl hydrogen peroxide complexes of Theorell (*11*) and of Chance (*12, 13, 14*). When catalase reacted with hydrogen peroxide, only one molecule of hydrogen peroxide was combined with one molecule of enzyme, forming the primary and secondary complexes. (*3, 4*) The primary catalase-peroxide complex could oxidize alcohol or nitrite in the process of its decomposition (*8, 9,* 10), while horse-radish peroxidase could oxidize the oxidizable substances by its secondary complex. (*14*)

Heppel and Porterfield (*15*) demonstrated that the coupled oxidation of nitrite was observed by catalase in the presence of a system of D-amino acid oxidase-amino acid, or of xanthine oxidase-hypoxanthine.

In this paper, the oxidation of ethanol or nitrite by catalase with hydrogen peroxide solution are mainly reported, and the conditions of the peroxidatic oxidation by catalase are discussed with reference to the mechanism of catalase reactivities.

EXPRIMENTALS

Crystalline beef liver catalase was prepared by the method of Dounce (*16*). This iron contents of catalase solutions were determined by the colorimetric method of Shinohara (*17*), by which the concentration of catalase was represented as "hematin iron" in this paper.

Qualitative Test of Peroxidatic Activity of Catalase—Neither benzidine- nor pyrogallol-color reaction was shown by this catalase preparation alone, at pH 7.0–3.0.

On adding the different concentrations of hydrogen peroxide solution, the peroxidatic activity of catalase was demonstrated by benzidine method in its lower concentration, as shown in Table I.

Table I

Peroxidatc Activity of Catalase at Various Concentrations of Hydrogen Peroxide

0.068 micromoles of hematin-iron beef liver catalase; 1 drop of benzidine-30% acetic acid solution; pH 5.0, 0.1 *M* acetate buffer.

Catalase hematin iron	Hydrogen peroxide	$\frac{[\text{Hematin iron}]}{[\text{Hydrogen peroxide}]}$	Color reaction
micromoles	*micromoles*		
0.068	2000	3.4×10^{-5}	−
0.068	1000	6.8×10^{-5}	−
0.068	500	1.4×10^{-4}	−
0.068	250	2.7×10^{-4}	−
0.068	100	6.8×10^{-4}	±
0.068	50	2.7×10^{-3}	+
0.068	10	6.8×10^{-3}	+

Varying the concentration of catalase, the peroxidatic reaction was observed by benzidine method, in its relatively high concentration compared with that of hydrogen peroxide, as shown in Table II.

The result of Table I showed a good agreement with that of Table II in respect to the ratio of [hematin iron]/[hydrogen proxide]; the benzidine color reaction was observed above the ratio of more than 10^{-3}.

Peroxidatic Activity of Catalase in Manometric Experiments—For the quantitative determination of the peroxidatic oxidation of certain substances by catalase and hydrogen peroxide, Warburg's manometric method was used. If catalase utilizes hydrogen peroxide to oxidize the

TABLE II

Peroxidatic Activity of Various Concentrations of Catalase

50 micromoles of hydrogen peroxide solution; 1 drop of benzidine-30% acetic acid solution; pH 5.0, 0.1 *M* acetate buffer.

Catalase hematin iron	Hydrogen peroxide	$\frac{\text{[Hematin iron]}}{\text{[Hydrogen peroxide]}}$	Color reaction
micromoles	*micromoles*		
0.27	50	5.4×10^{-3}	+
0.14	50	2.8×10^{-3}	+
0.027	50	5.4×10^{-4}	±
0.014	50	2.8×10^{-4}	−
0.0027	50	5.4×10^{-5}	−
0.0014	50	2.8×10^{-5}	−

oxidizable substances, the amount of oxygen evolved in the manometry will be decreased as much as the equivalents of the oxidation by catalase.

The manometric experiments were carried out under the conditions described in Table III. The last column of Table III showed the difference of oxygen evolution due to the peroxidadic oxidation of each oxidizable substance by catalase. Among them, ethanol, ascorbic acid, phloroglucinol, pyrogallol, and benzidine were calculated to be oxidized as much as $0.2—0.8\times10^{-6}$ equivalents. These amounts of calculated oxidation are, however, too little—about 10 micromoles of each substance being used—to give the evidence for the peroxidatic oxidation by catalase under the conditions in Table III.

The Peroxidatic Oxidation of Ethanol by Catalase and Hydrogen Peroxide—In order to add hydrogen peroxide at an arbitary rate, a syringe for injection (2 ml. or 5 ml.) was connected well with a needle made of glass, the end of which was made into a long capillary. This capillary was cut at different positions to give different calibres, through which hydrogen peroxide solution was added at different velocities into the reaction mixture.

Catalase, ethanol, and buffer were mixed in a round-bottom flask and cooled with ice.-water mixture. The syringe with a glass-needle was fixed in the vertical direction, filled with hydrogen peroxide solution, and the end of capillary was dipped enough into the reaction mixture. Thus the continuous addition of hydrogen peroxide was performed, so that it might be quite analogous to the mode of the formation

TABLE III

Peroxidatic Oxidation of Various Oxidizable Substances by Catalase and Hydrogen Peroxide

0.027 micromoles of hematin ironbeef liver catalase; 10 micromoles of each oxidizable substance; 0.3 ml. of 0.3% hydrogen peroxide (3 micromoles); 0.05 M buffer; incubation time for 30 minutes.

Oxidizable Substances	pH	Oxygen evolved (μl) System					
		(I)	(II)	(III)	(IV)	(III) − (IV)	[(III)−(IV)] + (I)
Ethanol	7.0*	0.5	0	233	212	21	21.5
Ascorbic acid	6.0*	−62	−98	238	163	75	13
Phloroglucin	6.0*	6	1	218	213	5	11
p-cresol (4 μM)	7.0*	13	15	131	144	−13	0
Pyrogallolol	6.8*	−81	−103	226	99	127	46
,,	6.0*	6	−2	216	214	2	8
,,	5.0†	8	0	225	225	0	8
,,	4.0†	8	2	227	231	−4	4
Benzidine	6.8*	−12	−21	237	210	27	15
,,	4.0†	3	4	228	219	9	12
Fumaric acid	7.0*	1	0	222	224	−2	−2

System (I) consists of oxidizable substance and catalase;
(II) consists of oxidizable substance alone;
(III) consists of catalase and hydrogen peroxide;
and (IV) consists of oxidizable substance, catalase, and hydrogen peroxide.
* Phosphate buffer; † Acetate buffer.

of hydrogen peroxide from the primary reaction in the coupled oxidation of Keilin *et al.* (*1*) or Heppel *et at.* (*15*).

After the completion of the addition of hydrogen peroxide, the remaining amounts of ethanol were measured by the following method:

Four ml. of Scott-Willson's reagent (NaOH-Hg(CN)$_2$-AgNO$_3$) was added into the round-bottom flask, in which the reaction had been carried out. Ethanol was distilled through a glass-tube with condenser, and exactly 10 ml. of the distillate was collected into a calibrated test-tube cooled well with ice. To this distillate, 10 ml. of Anstie's reagent (K$_2$Cr$_2$O$_7$-conc·H$_2$SO$_4$-mixture) was added, followed by dipping in a boiling water both for 10 minutes. After cooling, the intensity of the developed color was determined by Pulfrich's photomer with

S_{43}. Recovery of ethanol by this method was quite satisfactory, giving an average error of 0.2%.

In order to find the conditions under which catalase may react peroxidatically with hydrogen peroxide for the oxdation of ethanol, the time required for the addition of hydrogen peroxide was recorded as shown in Table IV.

TABLE IV

The Peroxidatic Oxidation of Ethanol by Catarase and Hydrogen Peroxide Solution

H_2O_2 added	Time required for addition	Ethanol oxidized	Ethanol oxidized per second	H_2O_2 added per second	Efficiency of H_2O_2 for enthanol oxidation*	Ratio of $\frac{\text{[Hematin-iron]}}{[H_2O_2 \text{ added per sec.]}}$
micromoles	*sec.*	*micromoles*	*micromoles*	*micromoles*	%	
1000	3	0	0	333	0	8.7×10^{-5}
	215	0	0	4.7	0	6.2×10^{-4}
	623	0.43	0.00068	1.6	0.043	1.8×10^{-2}
	2100	0.60	0.00029	0.48	0.06	6.1×10^{-2}
	3060	0.60	0.0002	0.33	0.061	8.8×10^{-2}
	4680	0.60	0.00013	0.21	0.062	1.3×10^{-1}
	7920	0.77	0.000097	0.13	0.075	2.2×10^{-1}
200	5	0.60	0.12	40	0.3	7.3×10^{-4}
	325	1.3	0.004	0.62	0.65	4.7×10^{-2}
	918	2.0	0.0022	0.22	1.0	1.2×10^{-1}
	2520	2.4	0.00095	0.079	1.2	3.6×10^{-1}
	3720	3.1	0.00083	0.054	0.5	5.4×10^{-1}
	5160	5.3	0.001	0.039	2.6	7.5×10^{-1}
	7680	5.6	0.00073	0.026	2.8	1.1
100	10	0.85	0.085	10	0.85	2.9×10^{-3}
	252	1.5	0.006	0.40	1.5	7.3×10^{-2}
	920	4.2	0.0046	0.11	4.2	2.6×10^{-1}
	2180	5.6	0.0026	0.046	5.7	6.3×10^{-1}
	4680	5.5	0.0012	0.021	5.7	1.4
	5520	6.5	0.0012	0.018	6.7	1.6
	7080	7.0	0.00099	0.014	7.1	2.1

$$\text{*Efficiency of } H_2O_2 = \frac{\text{[Ethanol oxidized/sec·]}}{[H_2O_2 \text{ added/sec]}}$$

0.029 micromoles of hematin iron beef liver catalase; 85 micromoles of ethanol; pH 7.2, 0.05 *M* phosphate buffer; 5±1°.

An insterest relationship between the addition-time and the amount of oxidized ethanol, was observed in Table IV: "The slower is the addition of hydrogen peroxide, and also the lower is the concentration of the added hydrogen peroxide, the more is the amount of ethanol oxidized."

The Peroxidatic Oxidation of Nitrite by Catalase and Hydrogen Peroxide—The concentration of the used nitrite was determined be permanganate titration in the presence of an excess amount of oxalic acid after oxidizing nitrite to nitrate with permanganate. The reaction was carried out under the same condition as in the experiments of the ethanol oxidation. For the determination of the amount of remaining nitrite after the reaction, the deproteinization was first done by adding 1 ml. of 0.5% $HgCl_2$ and 1 ml. of 2% Na_2CO_3. After filtration, Shinn's colorimetric method was employed (*17*).

In case of the nitrite oxidation, the similar results to those of the ethanol oxidation, were also obtained, as shown in Table V.

The Attempts to Detmonsrate the Peroxidatic Activity of Catalase by the Action of Inhibitor, Heat, Detergent, or Proteolytic Enzymes—

(a) It has been demonstrated by Chance that the simultaneous addition of the mixture of hydrogen peroxide and hydrocyanic acid forms the Catalase-(OOH) $(CH)_2$ complex (liver catalase preparation with 3 hematin-iron groups per enzyme molecule), in which to hematin-irons are combined with cyanide and the remaining one is combined with peroxide, and the combined peroxide is displaced by cyanide in about a minute.

Though this Catalase-(OOH) $(CH)_2$ complex shows no catalatic activity, the peroxidatic activity might be expected, because the general structure of hematin-iron-peroxide compound has been known to be highly active in horse-radish peroxidase reaction (*12*).

The mixtures of hydrogen peroxide and various amounts of hydrocyanic acid, ranging from 16 to 0.064 in $[HCN]/[H_2O_2]$ ratio, were added to catalase at pH 7.0 for the pyrogallol test. Howevere, no peroxidatic color reaction was observed.

(b) It was reported that when hemoglobin was treated with a high concentration of urea solution, its molecular weight became halved (*19*). If this is case with catalase, it might be expected that catalase molecule is distinergrated into peroxidase, because the latter has one hematin-iron group and about 1/3-1/5 of molecular weight of the former (*22*, *23*).

Catalase was incubated with 1, 2, or 4 *M* of urea solution for 3, 6,

TABLE V

The Peroxidatic Oxidation of Nitrite by Catalase and Hydrogen Peroxide
0.029 micromoles of hematin iron beef liver catalase, 17 micromoles of sodium nitrite; pH 7.2, 0.05 *M* phosphate buffer; 5±1°.

H_2O_2 added.	Time required for addition	Nitrite oxidized	Nitrite oxidized per second	H_2O_2 added per second	Efficiency of H_2O_2 for nitrite oxidation*	Ratio of $\frac{[\text{Hematin iron}]}{[H_2O_2 \text{ added per sec.}]}$
micromoles	*sec.*	*micromoles*	*micromoles*	*micromoles*	%	
1000	3	0.24	0.08	333	0.024	8.7×10^{-5}
	600	0.34	0.00056	1.7	0.033	1.7×10^{-2}
	2520	0.60	0.00024	0.39	0.062	7.5×10^{-2}
	5640	0.43	0.000076	0.18	0.042	1.6×10^{-1}
	6960	0.48	0.00007	0.14	0.050	2.1×10^{-1}
200	5	0.34	0.068	40	0.17	7.3×10^{-4}
	240	0.50	0.0028	0.83	0.34	3.5×10^{-2}
	1740	1.50	0.00086	0.12	0.72	2.4×10^{-1}
	5460	1.73	0.00032	0.037	0.87	7.8×10^{-1}
	8040	1.92	0.00024	0.025	0.96	1.2
50	10	0.99	0.099	5	2.0	5.8×10^{-3}
	360	1.56	0.0043	0.14	3.1	1.6×10^{-1}
	1560	2.64	0.0017	0.031	5.5	9.4×10^{-1}
	3780	2.71	0.00072	0.013	5.5	2.2
	6600	3.06	0.00046	0.0076	6.1	3.8

* The efficiency of $H_2H_2 = \frac{[\text{Nitrite oxidized/sec.}]}{[H_2O_2 \text{ added/sec.}]}$

12, 24, and 48 hours at 37°. But neither pyrogallol nor benzidine-test was demonstrated with thus treated catalase.

(c) The activity of catalase has been reported to be very labile to heat *(20)*, while that os peroxidase if rather stable, *(21)*. If therefore, the action of heat could bring about the denaturation of catalase protein to certain extent, to ensure the reduced activity as catalase, but rather to enhance that as peroxidase, the peroxidatic activity of thus treated catalase might be expected to appear.

The incubation of catalase at 37° and 60° for 10-180 minutes did not result the peroxidatic color reaction with pyrogallol- or benzidine-method, while the catalatic activity had been almost completely lost with the progress of the incubation.

(d) If any proteolytic enzyme could attack the catalase molecule and disintegrate it into three or four fractions, so that each of them many have still one hematin-iron group, it would be expected the appearance of the disintegrated substance with peroxidatic activity.

The experiments were carried out with papain at pH 7.2, pepsin at pH 4.0, and trypsin at pH 8.0, but neither pyrogallol nor benzidine color reaction was shown after the incubation time of 0.5-6 hours, while the catalatic activity had been reduced.

DISCUSSION

From the experiments with ethanol and nitrite, it is pointed out that hydrogen peroxide is utilized by catalase for the peroxidatic oxidation. Keilin and Hartree explained their coupled oxidation of ethanol by assuming the *nascent* state of hydrogen peroxide formed from the primary reaction (*1*). They also reported that for the coupled oxidation, a greet amount of catalase was required. Egami and Murakami could not demonstrate the peroxidatic oxidation of ethanol or nitrite by catalase, either with hydrogen peroxide or with the irradiated hydrogen peroxide treated with ultraviolet ray (*24*). In the coupled oxidation of ethanol (*1, 2*) or of nitrite (*14*), the conditions of the addition of hydrogen peroxide obtained in this paper, are considered to be satisfied, since a great amount of catalase was used, and the primary reaction system must have formed hydrogen peroxide quite continuously and in a very low concentration, so that the ratio of [hematin iron]/[hydrogen peroxide] would be more than 10^{-3} at any time. Therefore, it is concluded as follows: in coupled oxidation, the hydrogen peroxide available for the secondary oxidation by catalase is not necessary to be formed from the primary reaction, but it is rather essential that hydrogen peroxide should be added slowly and continuously in its lower concentration.

The catalatic and peroxidatic reactions of catalase and represented in the following equations:

Complex-I formation (the primary complex):

(Eq. 1) $$\text{Catalase-(OH)}_4+\text{HOOH} \xrightarrow{k_1=3\times 10^7 \text{ per mole per sec.}} \text{catalase-(OH)}_3\ \text{(OOH)-I}$$

Catalatic reaction:

(Eq. 2)

$$\text{Catalase-(OH)}_3\text{(OOH)-I}+\text{HOOH} \xrightarrow{k_4=3.5\times 10^7 \text{ per mole per sec.}} \text{Catalase-(OH)}_4+H_2O+O_2$$

Peroxidatic reaction:

(Eq. 3-a) $\text{Catalase-}(OOH)_3(OOH)\text{-I}+C_2H_5OH$

$\xleftrightarrow{k_4 1000 \text{ per mole sec.}} \text{Catalase } (OH)_4+CH_3CHO+H_2O$

(Eq. 3-b) $\text{Catalase-}(OH)_3(OOH)\text{-I}+NO^-{}_2$

$\xleftrightarrow{k_4=2000 \text{ per mole per sec.}} \text{Catalase } (OH)_4+NO^-{}_3+H_2O$

These values of the velocity constants were obtained by Chance with his "rapid flow method" (*5, 10*).

As the velocity of the reaction of complex-I with HOOH is much mole rapid than that with C_2H_5OH or with $NO^-{}_2$, the catalatic reaction (Eq. 2) would be expected to be almost complete before the peroxidatic reaction (Eq. 3-a or 3-b) takes place. Thus there would be no turnover of catalase in peroxidatic reaction, while in catalatic reaction there are a great number of turnovers. Thererfore, even if there is an excess amount of hydrogen peroxide in the reaction mixture, only one molecule of ethanol or nitrite would be oxdized per catalase molecule, while the remaining hydrogen peroxide unutilized for the peroxidatic reaction, is catalatically decomposed.

Assuming that the addition of peroxide in the oxidation of ethanol or nitrite in this paper is carried out at a constant velocity throughout the experiment, the amount of hydrogen peroyide added per second and the amount of ethanol or nitrite oxidized per second are calculated, and the ratio of [ethanol oxidized per sec.]/[hydrogen peroxide added per sec.] or of [nitrite oxidized per sec.]/[hydrogen peroxide added per sec.] are further calculated, as shown in Table IV and V, respectively. The ratio in Table IV and V, is represented in percentage, which will mean the efficiency of hydrogen peroxide to utilized for the peroxidatic reaction of catalase.

It is also observed that the amount of hydrogen peroxide utilized for the peroxidatic oxidation by catalase is dependent upon the amount of hydrogen peroxide added per second, in other words, upon the ratio of [catalase]/[H_2O_2] in reaction mixture, even when the same amount of hydrogen peroxide is added as a whole. (See the last two columns of Table IV and V.)

It has been known that catalase is predominantly an animal enzyme, While peroxidase is predominantly a plant enzyme, except for lacto-peroxidase from milk and verdoperoxidase from empyema fluid. The animal tissues contain much catalase compared with the amount of hydrogen peroxide which would be formed in cellular respiration, though there has been no direct evidence for the formation of hydrogen peroxide *in vito.* Considering the relationship between high content of catalase and very small amount of hydrogen peroxide formed in cellular enzymatic reactions, catalase would mainly play the role of the peroxidase *in vivo.*

SUMMARY

1. Crystalline beef liver catalase showed the peroxidatic activity with benzidine method, when the ratio of [hematin iron]/[hydrogen peroxide] was more than about 10^{-3}.

2. From manometric experiments, very little peroxidatic oxidation of ethanol, and pyrogallol by catalase was demonstrated.

3. In the experiments of the peroxidatic oxidation of ethanol or of nitrite by catalase, the rate of the added hydrogen peroxide was controlled by a glass-needle with an capillary. Various concentrations of hydrogen peroxide were added continuously through the capillary at different rates. It is observed that "the slower is the addition of hydrogen peroxide, and also the lower is the concentration of added hydrogen peroxide, the more is the amount of ethanol or nitrite oxidized."

4. The attempts to demonstrate the peroxidatic activity by attacking catalase molecule with heart, enzymes, urea, or cyanide, were unsuccessful.

The conditions of the peroxidatic reaction of catalase with hydrogen roxide obtained in this paper, were disaussed in relation to the reaction mechanisms of catalase.

Tanks are due to Prof. K. Kodama for his grateful advice and to Dr. S. Tanaka for his kind suggestions.

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The Journal of Biochemistry, Vol. 38, No. 1.

STUDIES ON NUCLEIC ACID METABOLISM
XIX. A SYNTHESIS OF 2-DESOXY-D-RIBOSE*, **

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(Received for publication, October 18, 1950)

The preparation of thyminose by hydrolyzing guanine nucleoside which was obtained by enzymic cleavage of thymonucleic acid, was achieved for the first time by Levene and London (*1, 2*) and it was stated from the analytical data that the sugar was a desoxypentose. The physical and chemical properties of the sugar were investigated by Levene and Mori (*3*), and in the first place compared with those of the synthetic D-xylodesose, excluding the identity of both. The L-ribodesyse was prepared according to Meisenheimer and Jung (*4*) by Levene, Mikeska, and Mori (*5*). They found that the synthetic L-ribodesose and its benzylphenylhydrazone showed rotations of the same value as thyminose and its benzylphenylhydrazone, respectively, the only difference being the direction of rotation. Also, the melting points were the same. So it was stated that the carbohydrate of thymonucleic acid was 2-desoxy-D-ribose. But it has remained unsettled for a long time to compare the natural sugar itself and its benzylphenylhydrazone with 2-desoxy-D-ribose and its benzylphenylhydrazone in melting point, optical rotations and other properties.

I have been also interested in its preparation and recently synthesized 2-desoxy-D-ribose by the following route:

Glucose→ calcium gluconate→D-arabinose→ D-acetobromoarabinose→D-diacetylarabinal→D-arabinal→2-desoxy-D-ribose.

D-Arabinose*** (m.p. 159°) was prepared from calcium gluconate exactly in the same way as that of Hockett and Hudson (*6*). According to the direction of Karrer and Becker (7), D-acetobromoarabinose (m.p. 130°) which was obtained by passing hydrogen bromide into the suspensint of D-arabinose in acetic anhydride, was reduced to D-diacetylarabinal with zinc dust at the low temperature and then deacetylated to D-arabinal (m.p. 81°, $[\alpha]_D^{22}=+196°$ in water) by dissolving it into barium hydroxide in the cold. The crystalline arabinal was converted to 2-desoxy-D-ribose by treating it with ice cold 1.0 N sulfuric acid for 2.5 hours. The synthetic 2-desoxy-D-ribose melted at 80°. The optical rotation was $[\alpha]_D^{22}=-56.5°$ in water and its benzylphenylhydrazone melted at 129°, and its optical rotation was $[\alpha]_D^{22}=-16.5°$ in pyridine.

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** This report was briefly presented before the 39th Research Committee of Vitamin B Complex at Tokyo, June 5th, 1950.

*** I wish to acknowledge my indebtedness to Prof. K. Sahashi for his advice in preparing D-arabinose.

These data are identical with those reported by Levene and Mori for the natural sugar.

EXPERIMENTAL

D-*Arabinose* (6)—A solution of 10.43 g. of barium acetate monohydrate in 30 ml. of water and a solution of 6.33 g. of ferrisulfate (Fe=22.49%) in 30 ml. of water were poured into a liter of water, 100 g. of calcium gluconate dihydrate was added ane heated to boiling with stirring. It was removed from the flame, and filtered on a Bûchner funnel precoated with carbon. To the clear amber-colored filtrate, 500 ml. of water was added and cooled to 35°. Then 60 ml. of 30 per cent hydrogen peroxide was added. Within a few minutes an evolution of carbon dioxide began and the temperature rose to about 55°. The reaction was completed in about half an hour as indicated by the sudden appearance of a dark purple color. After cooling to 40°, 60 ml. of peroxide was added again. The reaction took place similarly. The solution was filtered on a Büchner funnel precoated with carbon and concentrated in a vacuum to 125 ml. To this, 750 ml. of methanol were added with shaking and the resulting precipitate was filtered and washed with 150 ml. of methanol. To the clear filtrate 450 ml. of ether was added and after five minutes, the new precipitate was filtered off. The filtrate was concentrated in a vacuum to a thick syrup. It was dissolved in about 65 ml. of warm methanol, and cooled in a refrigerator for three hours. The crystalline D-arabinose was filtered and washed with methanol. The yield 24 g., m.p. 152-153°. It was recrystallized from methanol. Yield 18.2 g., m.p. 159°. The $[\alpha]_D^{20}$ at equilibrium was $-105°$.

D-*Acetobromoarabinose* (7)—This was prepared by passing hydrogen bromide into the suspension of 10 g. of D-arabinose in 50 ml of acetic anhydride, until saturated at −5°. The yellow colored solution was diluted with 150 ml. of cold chloroform and shaken 3 times with 50 ml. of ice water. The extract was washed with sodium bicarbonate and finally with water. The light yellow colored solution was dried with calcium chloride and evaporated under reduced pressure. From the thick syrup, crystalline D-acetobromoarabinos was obtained. The yield was 4.2 g. from 10 g. of D-arabinose. m.p. 130°C.

D-*Diacetylarabinal* (*7*)—120 ml. of 50 per cent acetic acid in a flask were cooled in an ice-salt mixture and 26 g. of zinc dust and then 12 g. of finely powdered D-acetobromoarabinose were added. The mixture was shaken with a mechanical stirrer at about −5°. After 3 hours the zinc dust was filtered and washed twice with 50 ml. of water. The filtrate was diluted with 500 ml. of water and then extracted four times with chloroform. The combined extract which amounted to 120 ml., was washed first with sodium bicarbonate, then with water. It was dried with sodium sulfate and concentrated to a syrup under reduced pressure. The syrup was fractionated at 5 mm. It boilded at 100-105°. Yield 4.0 g.

D-*Arabinal* (*7*)—8 g. of crystalline barium hydroxide was dissolved in 40 ml. of boiling water, and the solution were cooled with ice water mixture. When a small part of the solution was frozen, 4 g. of D-diacetylarabinal were added, and the mixture was shaken at about 5°. After 20 to 30 minutes a light yellow colored solution was obtained. The excess of barium hydroxide was removed as barium carbonate by passing carbon dioxide into the solution, and warming at 70° for about 15 minutes. The clear filtrate was evaporated under reduced pressure to a crystalline mass. It was extracted several times with 20 ml. of warm absolute alcohol and filtered. The filtrate was concentrated under reduced pressure (bath temperature at 60°) to a thick syrup which crystallized spontaneously or by seeding. It was recrystallized from boiling benzene. 1.8 g. of pure crystalline D-arabinal was obtained. m.p. 81°. $[\alpha]_{\mathrm{D}}^{22}=\frac{+3.92^\circ\times 100}{1\times 2}=+196^\circ$ (in water).

2-*Desoxy*-D-*ribose*—1.1 g. of crystallin D-arabinal was dissolved in 18.3 ml. of ice cold 1.0 *N* sulfuric acid, and the solution was allowed to stand at 0°. It became gradually faintly yellow, and after 2.5 hours a faint turbidity occurred accompanied by a flocculent precipitate. At this time the solution was soon neutralized with barium hydroxide and finally with barium carbonate. After removing the precipitate and barium carbonate, the clear and light yellow colored filtrate was concentrated to a thick syrup under reduced pressure without heating. Then we took up the syrup with absolute alcohol and treated it witn about twice its volume of dry ether. The resulting precipitate was filtered off and the filtrate was evaporated in a vacuum desiccator over phosphorus pentoxide and calcium chloride.

From the thick syrup after several days the 2-desoxy,D-ribose was obtained in crystalline form. This was washed with a small amount of cold normal propyl alcohol. m. p. 80°. The yield of the sugar was a little more than 0.3 g. The Sugar gave a strongly positive Dische and Kiliani test specific rotation was as follows:

$$[\alpha]_D^{22} = \frac{-1.13 \times 100}{1 \times 2} = -56.5^\circ \text{ (in water) } (3).$$

Asym-Benzylphenylhdrazone—0.3 g. of 2-desoxy-D-ribose was dissolved in 1.5 ml. of normal propyl alcohol, and 0.39 g. of freshly distilled benzylphenylhydraine was then added. After standing for three hours in a desiccator over calcium chloride and scratching occasionally, the mixture had changed to a crystalline mass. It was washed with ether and recrystallized from normal propyl alcohol (Photograph: Fig. 1).

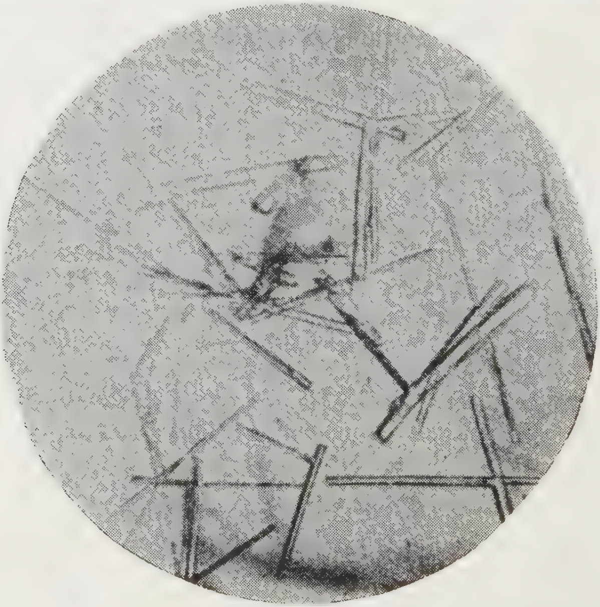

FIG. 1. Benzylphenylhydrazone of 2-desoxy-D-ribose

Yield 0.4 g. The substance melted at 129° (uncorrected).

$C_{18}H_{22}O_3N_2$ (314.2) Calculated, N 8.92; Found, N 8.94.

Its specific rotation was $[\alpha]_D^{22} = \frac{-0.33^\circ \times 100}{1 \times 2} - 16.5^\circ$ (in pyridine) (*3*).

I wish to express my thanks to the Fujisawa Pharm., Co., Ltd. for sending a sample of crude D-arabinose. I am also indebted to Mr. Ito in the Research Laaboratory, Takeda Pharm., Co., Osaka for measurement of optical rotations.

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The Journal of Biochemistry, Vol. 38, No. 1.

ACTION OF ALLOXAN ON ADRENALECTOMIZED RABBITS

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In the previous communication (*1*) the blood concentrations of glucose, lactate and pyruvate were examined in thh alloxan-diabetic rabbits. In spite of the remarkable increase of blood sugar, the values of blood lactate and pyruvate were normal in them. Janes and Friedgood (1945) (*2*) stated that in alloxan-treated rabbits adrenalectomy decreases the blood sugar to the normal value. Krahl and Cori (1947) (*3*) obtained the same results and also found that the diaphragma of alloxan-treated adrenalectomized rats utilized glucoge almost at the normal rate. Adrenalectomy alone is reported to cause in the normal rabits the decrease of blood sugar (*4, 5*) and liver glycogen (*5*), as well as the low level of blood lactate (*4, 6*). But these findings are not always verified. For instance, Cope and Corkill (*7*), ann Sawada (*8*) stated that blood sugar level was normal in the adrenalectomized rabbits, and Britton and Silvette (*5*) stated that the blood lactate was increased in them.

Epinephrine, the incretion of adrenal medulla, counteracts insulin, promoting hyperglycemia, but this action is temporary. On the other hand, adrenal cortex incretes some hormones, probably 11-hydroxy- or 11-keto- steroids, which promote hyperglycemia; this action is more persistent than epinephrine. At any rate it is an interesting problem how the blood sugar, lactate and pyruvate of adrenalectomized animals behave under alloxan injection, and the followings are the results of our experiments concerning this problem.

EXPERIMENTAL

Methods—Male and female rabbits, weighing about 2 to 3 kg., were used as stated in the previous report (*1*). Adrenalectomy was performed under the anesthesia of urethane, the adrenals of both sides having been taken off on the same day. After adrenalectomy, special care was taken with the room temperature and feeding. After three days further experiments were carried on, as the direct effect of the operation had disappeared by then.

The methods of alloxan treatment or glucose injection were the same as on the previous report (*1*), and also the methods of estimating blood sugar, lactate and pyruvate were the same.

RESULTS

Blood Sugar, Lactate and Pyruvate of Adrenalectomized Rabbits—Blood sugar, lactate and pyruvate of forty-five normal rabbits and eighteen adrenalectomized rabbits (on the fourth day aftar adrenalectomy) were compared. As Table I indicates, the values of the blood sugar, lactate and pyruvate on the fourth day after adrenalectomy were in the normal range, though somewhat lower values were observed in the maximum levels.

Blood Sugar, Lactate and Pyruvate in Adrenalectomized Alloxan-Treated Rabbits—Ten rabbits were treated with alloxan on the fourth day after adrenalectomy.* After 30 to 72 hours the blood sugar, lactate and pyruvate were determined. As Table I indicates, the values were the same as for the adrenalectomized rabbits. The remarkable elevation of blood sugar was not observed at all.

TABLE I

Concentration in Blood mg/dl	Average of 45 Normal Rabbits	Average of 18 Adrenalectomized Rabbits	Average of 10 Adrenalectomized Alloxan-treated Rabbits
Suger	127.5 ±3.9	105.6 ±4.3	105.3 ±9.3
Lactate	29.7 ±3.9	23.0 ±3.7	15.7 ±1.6
Pyruvate	3.05±0.44	2.11±0.20	2.05±0.21
S : L	7.28±0.70	7.11±1.25	7.59±1.15
S : P	54.4 ±3.9	57.6 ±6.1	57.8 ±9.6
S : P	8.9 ±0.5	10.2 ±1.0	8.0 ±0.8

Blood Sugar, Lactate and Pyruvate after Glucose Injection—Four adrenalectomized rabbits were injected with glucose, and the fluctuations of blood sugar, lactate and pyruvate were observed during seven hours. The blood suger attained the maximum valuc immediately after the injection and decreased slowly afterwards. After two hours the value was almost normal. Blood lactate and pyruvate took almost the same course as blood sugar (Fig. 1). This is almost the same finding as for normal rabbits previously reported (*1*). Only in one case was the recovery hf the blood sugar retarded, and even after seven hours the normal value was not attained, besides blood lactate and pyruvate took no increase in this case. The general condition of this rabbi was bad. It was weakened and asthenic.

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* The histological investigation was performed on the carcasses of these rabbits by Dr. Nagahama through courtesy of Prof. Akazaki of the Pathological Department, School of Medicine, Niigata Universyty. They showed the same degree of damages of Langerhans' islets as in the normal rabbits treated with alloxan.

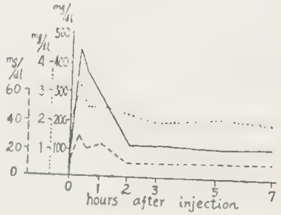

FIG. 1. Adrenalectomized rabbits injected with glucose (average of tree cases). (— blood sugar,---blood lactate ... blood pyruvate)

When alloxan-treated adrenalectomized rabbits were injected with glucose, the fluctuations of blood sugar, lactate and pyruvate were different than the above. The recovery of the elevated blood sugar values were retarded, and no increase of blood lactate or pyruvate value was observed in the hyperglycemic phase (Fig. 2. a). In some cases these values increased remarkably after three or five hours, and in these cases the general condition was so bad that death was brought about soon afterwards (Fig. 2. b).

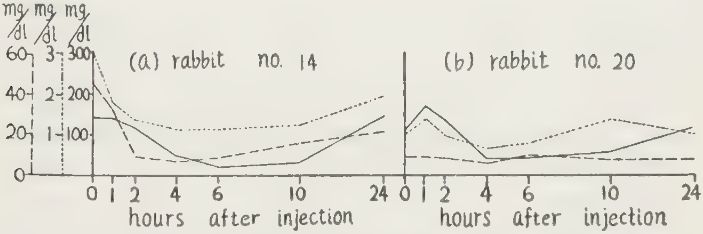

FIG. 2. Adrenalectoinized, alloxan-treated rabbits injected with Glucose. (— blood sugar, --- blood lactate, ... blood pyruvate)

Direct Influence of Alloxan Injection on Blood Sugar, Lactate and Pyruvate of Adrenalectomized Rabbits—If the adrenalectomized rabbits were treated with alloxan, the initial remarkable increase of blood sugar, which was almost always observed with normal rabbits, was not observed. The decrease of blood lactate and pyruvate was observed during the following hypoglycemic phase (in six out of eight cases) (Fig. 3. a). Sometimes (in two out of eight cases) hyperglycemia and slight elevation of blood pyruvate were observed, but in these rabbits adrenalectomy was incomplete or some accessory adrenal glands were found on the histological investigation of the carcasses (Fig. 3. b).

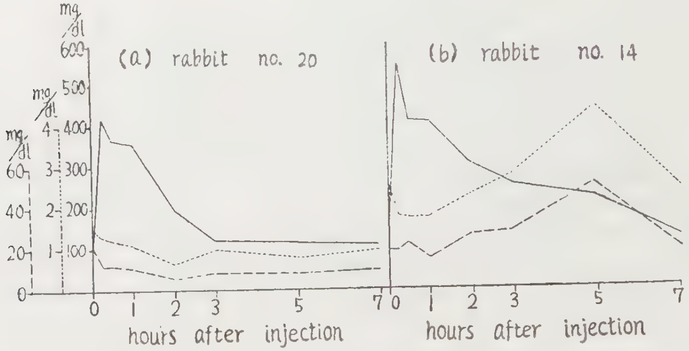

FIG. 3. Adrenalectomized rabbits injected with alloxan. (— blood sugar,--- blood lactate, ... blood pyruvate)

DISCUSSION

In the above experiments there were no differences of blood sugar, lactate and pyruvate found between the normal and the adrenalectomized rabbits. This finding on the blood sugar coincides with the report of Cope and Corkill (*7*), and Sawada (*8*). In these adrenalectomized rabbits no hyperglycemia was brought about by the treatment of alloxan. Janes and Friedgood (*2*), Krahl and Cori (*3*) stated that alloxan-diabetes was ameliorated by adrenalectomy. Also Long and his colleagues (1936) (*9*), (1937) (*10*) stated formerly that the adrenalectomy alleviated the diabetes of depancreatized animals. It is probable that in normal rabbits the incretion of adrenals is counteracted by the incretion of the islets. Howevere, if the rabbits were injected with glucose, distinct difference were found to have been brought about by the alloxan treatment, namely, the alloxan-treated adrenalectomized rabbits showed the retarded recovery of blood glucose and no elevation of blood lactate and pyruvate in the early phase, when the elevation of these was found in those rabbits not treated with alloxan. Thus, the alloxan-treatment causes damage to the conversion process from glucose to pyruvate or lactate even in adrenalectomized rabbits. The fluctuation of blood pyruvate and lactate ran parallel and there was no change in the equilibrium between pyruvate and lactate.

Colowick,, Cori, and Slein (*11*) stated that adrenal extracts inhibited the action of muscle hexokinase, and this inhibition was countracted by insulin. From their results it could be concluded that adrenalectomy might completely ameliorate the damage of the glucose metabolism brought about by the treatment with alloxan. but the inhibition of hexokinase was effected by the extracts of anterior pituitary (Price, Cori, and Colowick (*12*) and spleen (Broh-Kahn, and Mirsky (*13*)), too. Thus, the counteracting substances to insulin might not be completely removed by the adrenalectomy. Furthermore, many investigators hold the opinion that tee action of insulin is not only concerned with the hexokinase activity (*14–20*). Our results indicate that the conversion process of glucose to pyruvate and lactate is retarded when glucose is injected into the alloxan-treated adrenalectomized rabbits. The carbohydrate metabolism cannot be regulated completely in these animals, as the concrete endocrine system is lacking in them.

In the previous paper (*1*) the initial hyperglycemia and the following hypoglycemia were observed with normal rabbits after treatment with alloxan. If adrenalectomized rabbits were treated with alloxan, this initial hyperglycemic phase was not observed, and this finding coincides with the statement of Goldner and Gomori (*21*), Kirschbaum, Wells and Molander (*22*). Now, in these rabbits the decrease of blood lactate or pyruvate was observed in the following hypoglycemic phase, whereas no decrease or rather increase of these was observed in the hypoglycemic phase of alloxan-treated normal rabbits (*1*). This finding indicates that the elevation of blood lactate or pyruvate in this phase in alloxan-treated normal rabbits is probably due to the same cause as, or to the persisting influence of, the initial hyperglycemia.

SUMMARY

1. The alloxan-treatment of adrenalectomized rabbits caused almost no elevation of blood suger.

2. When alloxan-treated adrenalectomized rabbits were injected with glucose, the recovery of blood sugar level was somewhat retarded, and the elevation of blood pyruvate or lactate during the hyperglycemic phase, whicn was observed on the rabbits not treated with alloxan, was not observed-

3. As the previous communication (*1*) indicates, when normal rabbits were treated with alloxan, the initial hyperglycemia took place and no decrease of blood pyruvate and lactate was obierved during the following hypoglycemic phase. Now, when adrenalectomized rabbits were treated with alloxan, this initial hyperglycemia was not distinctly observed, and the blood pyruvate and lactate decreased during the following hypoglycemic plase.

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The Journal of Biochemistry, Vol. 38, No. 1.

SOME STUDIES ON THE GROUP SPECIFIC SUBSTANCE IN THE HUMAN SALIVA

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About the chemical nature of the group substance, the polysaccharide theory is now more plausible that the exact theory. Much works have been done on the group specific substance separated from pepsin and horse's saliva but few on that from human saliva. From this point of view, the author separated and purified the salivary effective substances of blood group of A, B, and O., and studied on their chemical and serological natures, the details of which will begin in this report, and the existence of the so-called blood group enzyme is postulated.

THE SEPARATION OF THE GROUP SUBSTANCES IN HUMAN SALIVA

The First Method—Collecting 180 ml. of saliva from the two persons belonging to the same blood group known beforehand, we made a centrifugation, took the upper transparent layer, acidified it with hydrochloric acid, and warmed it for 5 minutes in the boiling water-bath, and then the protein-coagulate settled out. After cooling it, we centrifuged it again. Taking its supernatant out, we solved 23 g. of anhydrous sodium acetate and added 450 ml. of absolute alcohol to it. After fully shaking, we left it over night, made a centrifuge, added 20 ml. of *N*-NaCl, to the deposit and after fully stirring it up, left it for several hours. We made again a centrifuge, cast aside the insoluble matter, added to the supernatant 2.8 g. of anhydrous sodium acetate and 50 ml. of absolute alcohol. After well mingling and shaking it, we left it until the polysaccharide settled out completely. The above operation was repeated once more (precipitation with sodium acetate and absolute alcohol.). We added 5 ml. of distilled water to the deposit thus obtained, stirred it up, left it till the following day to solve it as much as possible, made a centrifuge, cast aside the insoluble matter, took the supernatant, added to it glacial acetic acid as much as from 12 to 15 times, left it till the next day, and found some white precipitate at the bottom of the beaker. We exfoliated it, made a centrifuge of it, washed it with a small amount of alcohol, and dried it in a desiccator. Yield 7 mg.

The Second Method—Besides the first method we had the second method. We collected the saliva from one person of secreting type A. Deproteination was performed with Sevag's method. Collecting 150 ml. of saliva, we filtrated it through a gauze, after a centrifuge, took the upper transparent layer, acidified it with sulphuric acid and heated it for 5 minutes in the boiling water-bath. After cooling it, we made a centrifuge again, took the supernatant, added 19 g. of anhydrous sodium acetate and 375 ml. of alcohol to it and left it until the following day. The deposit was centrifuged out, solved in 13.6 ml. of *N*-NaCl, mixed well and left until the following day. The insoluble matter was again centrifuged out, and cast aside. We added to the supernatant 1.98 g. of anhydrous sodium acetate and 34 ml. of alcohol. After well mixing and shaking it, we settled it again. The same operation was repeated once more (*N* NaCl, anhydrous sodium acetate, absolute alcohol).

Solving the deposit in 14 ml. of water, we removed the insoluble matter by centrifuge, made pH 4.8 with acetic acid, added to it 28 ml. of chloroform and 0.93 ml. of *iso*-butyl alcohol according to the Sevag's method, put a small glass ball in it, shook severely for 5 hours, using an electric agitator, and then laid it quietly in a separating funnel to separate it into the upper transparent chloroform layer. And we repeated this Sevag's procedure 3 times, and could collect 42 ml. supernatant in all. Dialyzing it through a collodium membrane for 48 hours against running water, removed it on a watch-glass, evaporated at a low temperature until it became 3.5 ml. and left it till the next day. After adding 35 ml. of acetone to it, we separated the deposit by centrifuge, washed it with ether and alcohol, and dried it in an desiccator. Yield 6 mg.

The material separated with the first and the second methods is apparently pure white, amorphous, hygroscopic powder, soluble in water, and insoluble in acetone, alcohol, and ether. Protein reaction sulfosalicylic acid test, and picric acid test) negative, Molisch reaction positive.

The yield was not fixed every time, and extremely small amount, so we collected it in succession.

MICROELEMENTAL ANALISIS OF GROUP SUBSTANCE

We collected materials from group A saliva of secreting type (Toda), which inhibition titer is 1 : 270, from group B saliva of secreting type (Ryu), which the inhibition titer is 1 : 810, from group B saliva of non-secreting type (Kawagoe), and group O saliva of non-secreting type, (Decided with anti group O cattle serum) (Nagasaka) each. Then separating and purifying carbohydrate-like-substances from them, we made the elemental analysis and got the following data.*

(Hearty thanks to the members in the micro-analytical department of the Takeda Chemical Laboratory, whom we asked to analyze them).

Substance separated from group A saliva (Toda) (Inhibition titer 1 : 270)

CH.	Substance	CO_2	H_2O	C%	H%
	1. 636 mg.	2. 600 mg.	1. 050 mg.	43. 50	7. 21
N.	Substance	N_2		N%	
	3. 325 mg.	0. 157 ml. (11°, 768 mm.)		5. 74	

Sugar content 47 ·5% (Hagedorn-Jensen).

Substance separated from group B saliva (Ryu) (Inhibition titer 1 : 810)

CH.	Substance	CO_2	H_2O	C%	H%
	3. 200 mg.	4. 963 mg.	1. 950 mg.	42. 30	6. 82
N.	Substance	N_2		N%	
	1. 564 mg.	0. 088 ml (9°, 762 mm.)		6. 83	

Suger content 46. 8% (Hagedorn-Jensen)

Substance separated from group B salive (Kawagoe), so-called non-secreting type.

CH.	Substance	CO_2	H_2O	C%	H%
	3. 340 mg.	3. 160 mg.	1. 250 mg.	25. 80	4. 19
N.	Substance	N_2		N%	
	2. 743 mg.	0. 106 mg. (10°, 762 mm.)		4. 67	

Substance separated from group O saliva (Nagasaka), so-colled non-secreting type (Decided with anti group O cattle serum)

CH.	Substance	CO_2	H_2O	C%	H%
	2. 765 mg.	1. 860 mg.	0. 885 mg.	18. 35	3. 58
N.	Substance	N_2		N%	
	1. 385 mg.	0. 019 ml. (11°. 768 mm.)		1. 66	

In order to compare the data of analysis above shown with those o blood group substances obtained from several materials by various workers, we show the following table.

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* Micro elementary analysis was performed on the group substance obtained according to the first method only.

Author	1933 Brahn and Schiff	1935 Freuden berg	1936 Land-steiner	1936 Land-steiner	1938 Goebel	1939 Goebel, Beeson, Hoagland,	1945 Kawagoe			
Material	Pepsin Group A	Pepsin Group A	Pepsin Group A	Horse Saliva Group A	Neo-pepton Group A	Pneumo-coccus GroupXIV Group A	Human Saliva			
							Inhibiting titer 1 : 270 Group A	Inhibiting titer 1 : 810 Group B	So-called non-secreting type Group B	So-called non-secreting type Group O
C %	42. 8	45. 5	46. 88	44. 56	46. 68	44. 98	43. 50	42. 30	25. 80	18. 35
H %	8. 41	5. 7	6. 62	6. 91	6. 53	6. 46	7. 21	6. 82	4. 19	3. 58
N %	4. 63	5. 4	6. 16	7. 08	5. 85	2. 08	5. 74	6. 83	4. 67	1. 66
S %			0. 08							
P %			0. 10							
Acetyl			9. 95							

SEROLOGICAL STUDY ON THE HUMAN SALIVA AND THE SEPARATED GROUP SUBSTANCES

Experimental materials:

1) As for the human saliva, we collected saliva naturally secreted from a definite person whose blood group was known before, filtrated it through the filter paper, centrifuged it, and supplied the supernatant or the 10 times diluted fluid of saliva to the test substance.

2) As for the group substances, we used those supernatant which were separated and purified as described above, and made 1000 times diluted solution. When necessary, we made 100 to 150 times dilution as the original fluid.

3) As for the α and β standard sera, we collected and separated them from persons whose blood group were known before. And as for the anti group O serum, we collected and separated from cattle blood, made inactive by heating at 56° for 30 minutes, added 5% phenol (as much as one tenth of the volume of serum) to it, kept it in an ice-chamber and then used tlem by diluting it 3 times immediately before the experiments.

4) As for the blood cells, we collected them from the persons whose blood group was already known, prevented coagulation by adding sodium citrate, washed them 2 or 3 times with physiological saline solution, made 1% blood corpuscles suspension, and used the fresh blood corpuscles within a day to avoid pseudoagglutination.

Experimental method:

1) In order to determine the antiagglutination activities we used the hallow deck glass method and used the following signs to describe the results of the experiment.

(+++) shows the same agglutination as that of the control.

(++) shows the moderate agglutination, which is a little weaker than that of the control.

(+) shows the slight agglutination, but still we can see with the naked eye.

(±) shows those which we cannot see with the naked eye, yet we can see with a microscope.

(−) shows that no agglutination can be seen either with the naked eye or with a microscope, namely it shows the perfect inhibition.

2) For testing group A saliva or substance, we used serum α as

absorbent, and human blood cell of group A. For group B saliva or group B substance, we used serum β as absorbent, and human blood cell of group B.

We took 12 test tubes, into the first tube put the 10 tims diluted solution of the test substance, and then made progressively three fold dilution till the eleventh tube, and in the last twelfth (control) the physiological saline solution. Then we put a drop of them into the curvature of hallow deck glass, added a drop of 3 times diluted standard serum, let it be absorbed for 30 minutes and again to each added a drop of 1% blood cell suspension. After leaving it quietly for 30 minutes we examined the inhibiting effect.

RESULTS

The Inhibiting Activity of the Human Saliva (group A and group B) against the Standard Serum—As it will be sown in the following table, the per-

TABLE II

Inhibitory Effect of Human Saliva (A. B) Against the Standard Serum

Material		Group A Saliva				Group B Saliva			
Dilution	Names	Toda	Memoto	Shitomi	Yama-shita	Ryu	Kaku	Tagami	Kawa-goe
1	1 : 10	−	−	−	−	−	−	−	⧺
2	1 : 30	−	−	−	−	−	−	−	⧺
3	1 : 90	−	−	−	+	−	−	−	⧺
4	1 : 270	−	+	−	+	−	+	−	⧺
5	1 : 810	±	+	+	+	−	+	+	⧺
6	1 : 2430	+	+	+	⧺	+	+	+	⧻
7	1 : 7290	+	⧺	+	⧺	+	⧺	⧺	⧻
8	1 : 21870	+	⧺	⧺	⧺	+	⧺	⧺	⧻
9	1 : 65610	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧻
10	1 : 196830	⧺	⧻	⧺	⧻	⧺	⧻	⧻	⧻
11	1 : 590490	⧻	⧻	⧻	⧻	⧻	⧻	⧻	⧻
12	0	⧻	⧻	⧻	⧻	⧻	⧻	⧻	⧻

Degree of agglutination
− = No agglutination
± = Faint agglutination
+ = Slight agglutination
⧺ = Marked agglutination
⧻ = Slight egglutination

fect inhibition of group A saliva was seen in case of Toda till 1 : 270, Nemoto till 1 : 90, Shitomi till 1 : 270, Yamashita till 1 : 30, and in case of group B saliva Ryu till 1 : 810, Kaku till 1 : 90, Tagami till 1 : 270; but Kawagoe saliva showed no inhibition at all, even with the original fluid.

The Inhibitory Effect of the Separated Substance from Human Saliva against the Standard Serum—As the starting material for separation we selected for group A the spittle of Toda, the inhibiting effect of which was thought most forcible in the above test (1 : 270) and for group B, Ryu, whose inhibiting effect was 1 : 810. On separating and purifying carbohydrate, we compared the effect of inhibition upon the agglutination, by use of these substances diluted 1000 times as much as the original fluid.

As shown in the following table, the substance separated from group A spittle (Toda's) showed the perfect inhibition till 1 : 27000, and the one separated from group B spittle (Ryu's) showed it till 1 : 243000.

TABLE III

Inhibitory Effect of Isolated Group Substances (A. B.) From Human Saliva Against the Standard Serum

Dilution	Material		Group A (Toda)		Group B (Ryu)	
			Saliva	Separated Substance	Saliva	Separated Substance
1	1 : 10	(1×10^3)	−	(−)	−	(−)
2	1 : 30	(3×10^3)	−	(−)	−	(−)
3	1 : 90	(9×10^3)	−	(−)	−	(−)
4	1 : 270	(27×10^3)	−	(−)	−	(−)
5	1 : 810	(81×10^3)	±	(±)	−	(−)
6	1 : 2430	(243×10^3)	+	(+)	+	(−)
7	1 : 7290	(729×10^3)	+	(+)	+	(±)
8	1 : 21870	(2187×10^3)	+	(+)	⧺	(+)
9	1 : 65610	(6561×10^3)	⧺	(⧺)	⧺	(+)
10	1 : 196830	(19683×10^3)	⧺	(⧺)	⧺	(⧺)
11	1 : 590490	(50049×10^3)	⧻	(⧻)	⧻	(⧻)
12	0		⧻	(⧻)	⧻	(⧻)

The Rise and Fall of the Inhibiting Effect in the Course of Separating and Purification—We separated the protein from group A and group B saliva (each of 80 ml.) through acidification with HCl. Then, centrifuging the precipitate, (I) we settled it out, took 0.1 g. out of it (dried moderately), extradted with 5 ml. of physiological saline solution, made it solution I, to the rest dry substance (I) added 10 ml. of physiological saline solution, agitated and dissolved as much as possible. And after centrifugation we removed the supernatant. The undissolved residue (II) was half dried, 0.1 g. of it was extracted with 5 ml. of the physiological saline solution and was named II-solution.

We added to the supernatant of the precipitate (I) 10 g. of anhydrous sodium acetate and 200 ml. of absolute alcohol, and the precipitate (III) thus obtained was separated by centrifugation. The moderately dried substance of it was dissolved in 5 ml. of physiological saline solution (solution III).

The rest of precipitate (III) was treated with 10 ml. of *N* NaCl and the undissolved matter was centrifuged off. The supernatant was again precipitated with anhydrous sodium acetate and absolute alcohol. The precipitate (IV) settled thereby was separated by centrifugation, and 0.1 g. of it was dissolved in 5 ml. of physiological saline solution (solution IV).

The rest of precipitate IV was treated with 5 ml. of absolute alcohol (solution V) and the undissolved was centrifuged off. Then we left each solution in an ice-chamber for 24 hours, made 10 times dilute solution for use from them as the original solution, made successively 3 times dilution and tested the inhibiting activity. The result was as follows:

Group A Saliva

Solution I	inhibition titer 1 : 10
Solution II	inhibition titer 1 : 10
Solution III	inhibition titer 1 : 90
Solution IV	inhibition titer 1 : 90
Solution V	no inhibitory activity.

Group B Saliva

Solution I	inhibition titer 1 : 10
Solution II	inhibition titer 1 : 10
Solution III	inhibition titer 1 : 90
Solution IV	inhibition titer 1 : 90
Solution V	no inhibitory effect.

Thus the powerful part of the group substance is soluble in water but not precipitated with acid and insoluble in alcohol, and the exceedingly powerful inhibiting activity seemed to be in the polysaccharide fraction.

The Inhibiting Effece of Group O Saliva and Its Separated Substance—In 1941 Witebsky and Klendschoj (*1*) said that there was anti group O serum. So we collected sera from six cattles for testing, and observed that they remarkably aagglutinated the human group O blood cell, and we applied these in testing group O human saliva and its separated substance serologically. The anti group O serum used here was the anti group O cattle serum No. III, and we used it diluting to 1:3. In this test, we saw, though the examples were a few, that those cattle's sera, which did not agglutinate human group O blood cell, did not agglutinate other group A, B, and AB human blood cell.

TABLE IV

Agglutination Test of Cattle Serum and Human Group O Blood Cell

Agglutinant	Blood Cell Used for Agglutination Test			
	Group G Human Blood Cell	Group A Human Blood Cell	Group B Human Blood Cell	Group AB Human Blood Cell
I Anti group O Cattle serum	−	−	−	−
II Anti group O Cattle serum	+++	+++	+++	+++
III Anti group O Cattle serum	+++	+++	+++	+++
IV Anti group O Cattle serum	−	−	−	−
V Anti group O Cattle serum	+++	+	+	++
VI Anti group O Cattle serum	+++	+++	+++	+++

According to the result of the test for the inhibiting action of group O saliva and its separated substance, Koyama's saliva showed the complete inhibition till 1:30. Abe, Nagasaka, and Hosoda's showed no domplete inhibition. We separated the carbohydrate like substance from Koyama's saliva and this showed the perfect inhibition till 1:1000. Hereupon we can say that group O substance inhibits the agglutination of group O blood cell by the anti group O cattle serum, and in the case of group O, also, the so-called excretive and in-excretive types seem to be there.

Excretive Type and In-excretive Type—The amount of the group substance in the human saliva differs remarkably according to the individual. Since Schiff and Sasaki (*2*) advocated in 1932, it is possible to divide it into two, excretive and in-excretive groups according to the strength of inhibition on the specific group agglutination. Many followers studied and discussed intensively in some degree, but the origin of the excretive element is not yet known and we can find no definite opinion about it. Nomura (*3*), especially, advocated in 1934 that the group substance through little was still excreted, even in such a case as belongs to the so-called in-excretive type of Schiff and Sasaki (*3*), and that it was reasonable to devided it into the weak excretive group and the strong excretive type. Hereupon, we tested the inhibiting activity of the carbohydrate-like substance separated from Kawagoe's group B, and Nagasaka's group O saliva, both of which having been thought to be in-excretive, and we could find no inhibiting effect of these two separated substances in each to 1 : 1000 diluted solution, but in from 100 to 150 times diluted fluid cf Kawagoe's substance we saw complete innibition and with Nagasaka's substance it showed (±) inhibition in 1 : 100 to 150 dilution.

This proves that the group substance is contained and excreted in a small quantity even in the salive hitherto having been thought to be the in-excretive group.

By this I think rather, in analogy with so called blood sub-type, that there may exist some such a sub-type also in the case of saliva according to the strength based upon the individual difference.

The Difference of Inhibition According to the Separating Methods (The First and The Second Methods)—Though the insolubility of group substance increased with the elapse of time after having been prepared by both methods, the substance separated by the 1 st method could be perfectly dissolved by heating at 100° for 30 minutes, but that separated by the 2 nd method showed a little difficulty in dissolving. And the effect of inhibition differs, and if we compare those separated from the same group A saliva, the titer of the complete inhibition of the group substance separated by 1 st method is 1 : 27000, while the one separated by 2 nd method is 1: 9000, and as the operation of the 1 st methnd is much simpler than the 2 nd method, we dare say the 1 st method is better for purification and separation of the group substance.

The Cetection of the Group Substance in Several Bacterial Polysaccharides—Considering that there is the same group (as the group A) contained in bacterial polysaccharide especially in that of pneumococcus, we tested the existence of the blood group several bacterial polysaccharides separated and puaified in this laboratory.

According to the result of the experiment, we found no blood type in the polysaccharides of typhoid bacillus (Group No. 35, group No. 36), para C, polysaccharide from sputum of an open tuberculosis patient, *Achorin gypseum*, *Trichophyton violaceun*, *Microsporon lanosum etc.* And we separated the polysaccharide from *Pneumococcus* group XIV, and could prove the existance of group specificity in it agreeing with Goebel.

PHYSICAL INFLUENCES UPON SALIVA AND THE SPECIFIC GROUP SUBSTANCE

The Influence of Rays and Others—we radiated various kinds of rays, such as ultra-short waves, ultra-sound waves and *etc.* to the group A and B saliva (10 times) and to the group A and group B, separated specific substances (1000 times diluted solution) and compared them with those which were not radiated as the control to determine the reduction of the inhibitory effect. As for the applying condition, we radiated ultra-red rays from 25 cm. for 60 minutes; ultra-violet from 50 cm. for 60 minutes (we used a quartz test tube here), and as for Röntgen rays, we used the Siemen's Apparatus; Stabilivolt 140 KW, as a filter 0.3 mm. of copper plate plus 1 mm. of aluminium plate; radiating time 54 minutes, amount 600 V.

As for the ultra-short waves, we used Ultratermapparatus; wave length 6 m., filamentvoltage 18.; Schliephake electrode diameter 15 cm.; the distance between both electrodes 30 cm.; radiating time 60 minutes.

As for the ultra-sound waves, Kubota-manifactory's apparatus; 750 kilocycle in a second; radiating time 30 minutes.

According to the results of the experiment made here under the conditions above mentioned, the perfect inhibition was seem with all rays, namely the same as the control and we could see no change at all (we omitted the data.) And after leaving group A saliva and its group substance, group B saliva and its group substance at the room temperature for a week, we tested again under the conditions above mentioned, and though we could find no change in the case of ultra-violet, Röntgen rays, ultra-short waves, and ultra-sound waves, in the case of ultro-red rays, we saw some change of inhibition. Namely, when we radiated the ultra-red rays to group A saliva having an inhibition titer of 1 : 270, the activity was reduced to 1 : 90, and in the separated group A substance having the inhibition 1 : 27000; the activity was reduced to 1 : 900. At the same time we saw in group B saliva having the inhibition of 1 : 810, the activity was reduced to 1 : 270, and in group B substance having the inhibition of 1 : 243000, the activity reduced to 1 : 27000 (when we radiated ultra-red rays, the temperature was from 27° to 28°; the test of inhibition was done just before and immediately after the radiation, and as the agglutinant we used the blood cell suspension of the same origin.

From this result, we cannot tell why there was no change when we radiated the rays on the fresh substance, while there was reduction of inhibition when we radiated on the substance which experienced a week elapsion.

The more precise examination will be necessary on this.

The Influence of Cooling—Using the specific chamber at the Dairen Railway Research Institute, we cooled group A and group B saliva (1 : 10 dilution) and solutions of group A and group B substance (1 : 1000 dilution) respectively at 0°, −10°, −20°, −30°, −40°, −50°, −60°, −65°, and after 60 minutes we again liquefied them and tested the inhibition, resulting in finding there still remained complete inhibition, even at −65°.

The result at −65° is as follows:

The complete inhibition
in group A Saliva 1 : 30
in group A separated Substance 1 : 9000
in group B separated Substance 1: 27000

Every one of them was quite the same with that of the control, and there was no influence by cooling.

The Influence of Heating—In 1929, Schiff (*4*) said that there was seen no change of the group substansc from human blood cell by heating at 100° for several hours. In 1940, Witebsky and Klendschoj (*5*) disolved group A substance from peptone in physiological saline solution and boiled it for 15 minutes, but there was no change at all. In 1928, Yoshida (*6*) heated oral mucous membrane for 30 minutes at 80° but it did not lose its specificity, and in 1934, Kamimichi and Masaki- (7) said that there occurred on change of group substance by heating at 100° for 30 minutes.

So we performed the following experiment: We closed up each of a definite amount of group A and group B saliva and the group substance in an ampulla, put them into a water both, heated each of them at 60°, 65°, 70°, 75°, 80°, 85°, 90°, 95°, and 100°, tested the inhibiting action and saw that every one still had the same inhibition as the control. So we again investigated how much reduction of inhibiting effect could be seen when we heated them at 100° for 60 minutes. By this result we knew the inhibiting action was quite the same with that of the unheated one.

In order to test again at higher temperatures, on one hand we took 3 ml. respectively from group A and group B saliva in the test tubes, heated them to evaporate, and on the other hand we put the dry group substance itself into the test tubes, heated each of them at 100°, 150°, 200°, 250°, and 300° for 60 minutes in the oil bath, added 3 ml. of physiological saline solution (the same volume with group A and group B saliva before the evaporation) to the evaporated saliva, dissolved the group substance to 1: 1000, and lastly after leaving them 24 hours determined the change of inhibiting effect comparing with the inhibition of the control. As its results group A salive showed, after it had been heated at 100° for 60 minutes, the same inhibiting activity as it showed in ampulla, and it had the same activity with that of the control diluted to 1: 30.

At 150° for 60 minutes it lost the complete inhibition and when it was diluted from ten to thirty times it showed the inhibition of (+). At 200°, 250°, and 300°, it completely lost the inhibiting effect.

In group A substance it showed the same complete inhibition of 27000 times as the control. When heated at 100° for 60 minutes, and at 150° for 60 minutes the inhibition titer reduced to 1 : 9000, at 200° for 60 minutes, it reducted to 1: 1000 and at 250° for 60 minutes it lost the complete inhibition. When it was 1000 times, it showed the inhibition of (+), and at 300° for 60 minutes it completely lost the inhibiting action.

In group B saliva, at 100° for 60 minutes it showed the same degree of the complete inhibition at the dilution of 270 times as the control, at 150° for 60 minutes it was reduced to 10 times, and at 200°, 250°, and 300° for 60 minutes, it completely lost the inhibiting force.

In group B substance, at 100° for 60 minutes, it showed no change at all as the control showing the complete inhibition of 243000 times, at 150° for 60 minutes it was reduced to 1000 times, at 250, 60 minutes

Table V

The Influence of Heating on Group A Saliva and Separated Substance

Blood Group			Group A											
Material			Saliva						Separated Substance					
Dilution		Condition	Cont-rol	100° 60min.	150° 60min.	200° 60min.	250° 60min.	300° 60min.	Cont-rol	100° 60min.	150° 60min.	200° 60min.	250° 60min.	300° 60min.
1	1 : 10	(1×10³)	−	−	+	+++	+++	+++	(−)	(−)	(−)	(−)	(+)	(+++)
2	1 : 30	(3×10³)	−	−	+	+++	+++	+++	(−)	(−)	(−)	(++)	(++)	(+++)
3	1 : 90	(9×10³)	±	±	++	+++	+++	+++	(−)	(−)	(−)	(++)	(+++)	(+++)
4	1 : 270	(27×10³)	+	+	++	+++	+++	+++	(−)	(−)	(+)	(+++)	(+++)	(+++)
5	1 : 810	(81×10³)	+	+	+++	+++	+++	+++	(+)	(+)	(++)	(+++)	(+++)	(+++)
6	1 : 2430	(243×10³)	++	++	+++	+++	+++	+++	(++)	(++)	(+++)	(+++)	(+++)	(+++)
7	1 : 7290	(729×10³)	++	++	+++	+++	+++	+++	(++)	(++)	(+++)	(+++)	(+++)	(+++)
8	1 : 21870	(2187×10³)	+++	+++	+++	+++	+++	+++	(++)	(++)	(+++)	(+++)	(+++)	(+++)
9	1 : 65610	(6561×10³)	+++	+++	+++	+++	+++	+++	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)
10	1 : 196830	(19683×10³)	+++	+++	+++	+++	+++	+++	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)
11	1 : 590490	(59049×10³)	+++	+++	+++	+++	+++	+++	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)
12	0		+++	+++	+++	+++	+++	+++	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)

TABLE VI

The Influence of Heating upon Group B Saliva and Separated Substance

Bloop Group			Group A											
Material			Saliva						Separated Substance					
Dilution		Condition	Control	100° 60min.	150° 60min.	200° 60min.	250° 60min.	300° 60min.	Control	100° 60min.	150° 60min.	200° 60min.	250° 60min.	300° 60min.
1	1 : 10	(1×10³)	−	−	−	+++	+++	+++	(−)	(−)	(−)	(−)	(−)	(+++)
2	1 : 30	(3×10³)	−	−	+	+++	+++	+++	(−)	(−)	(−)	(++)	(++)	(+++)
3	1 : 90	(9×10³)	−	−	++	+++	+++	+++	(−)	(−)	(−)	(++)	(+++)	(+++)
4	1 : 270	(27×10³)	−	−	++	+++	+++	+++	(−)	(−)	(+)	(++)	(+++)	(+++)
5	1 : 810	(81×10³)	+	+	+++	+++	+++	+++	(−)	(−)	(++)	(+++)	(+++)	(+++)
6	1 : 2430	(243×10³)	++	++	+++	+++	+++	+++	(−)	(−)	(++)	(+++)	(+++)	(+++)
7	1 : 7290	(729×10³)	++	++	+++	+++	+++	+++	(+)	(+)	(++)	(+++)	(+++)	(+++)
8	1 : 21870	(2187×10³)	+++	+++	+++	+++	+++	+++	(++)	(++)	(++)	(+++)	(+++)	(+++)
9	1 : 65610	(6561×10³)	+++	+++	+++	+++	+++	+++	(++)	(++)	(+)	(+++)	(+++)	(+++)
10	1 : 196830	(19683×10³)	+++	+++	+++	+++	+++	++	(+++)	(+)	(++)	(++)	(+++)	(++)
11	1 : 590490	(59049×10³)	+++	+++	+++	++	+++	+++	(+++)	(+++)	(+++)	(+++)	(+++)	(++)
12	0		+++	+++	+++	+++	+++	+++	(+++)	(+++)	(+++)	(+++)	(+++)	(−)

it still showed 1000 times and showed the complete inhibition, and at 300°, for 60 minutes it quite lost the inhibiting force.

Judging from the above mentioned, the group substance in salive received no influences at 100° for 60 minute. and at 150° for 60 minutes the activity seemed to be gradually reduced, but in saliva at 200° for 60 minutes, with the purified substance at 300° for 60 minutes, inhibiting action was prefectly lost.

From the fact above written, we many well say there will be some difference in reduction of the inhibiting action according to whether it exists in dissolved state or in dry.

THE INFLUENCE OF PRESERVATION

As to how long is the specific inhibiting substance detectable, Shirai (*8*) in semen after 7 months, and Fujita (*9*) in semen spots after 6 months, could observed that the group specificity was preserved. Haraguchi (*10*) took spittle and piece of epidermis of oral mucous membrane adhered on a piece of cloth, and leaving it in a certain place for a certain time, and tested it for group substance. As its result he saw that both preserved the specificity for 80 days.

Haraguchi (*11*) was sure to prove the existance of the specifity in urine spots and a piece of dried oral mucous membrance for more than 400 days.

At this, in order to see the influence of the preservation of human saliva and the purified group substances upon its inhibiting power, we kept the group A saliva (10 times) (Nemoto's), the separated group A substance (1:1000 solution) (Toda's), the group B saliva (1: 10 Solution (Kaku's) and the separated group B substance (1:1000 solution) (Ryu's) in the sterilized ampulla, and at the same time, we preserved the group A saliva (1:10) (Toda's) and the group B saliva (1:10) (Kaku's) each in the open test tubes at the room temperature. After 200 days elapsed we tested the four groups and found that the titer of the complete inhibition reduced: That is, in the group A saliva inhibition of 1: 90 on the very first day was reduced to 1:30, in the group B saliva inhibition of 1:270 on the very first day was reduced to 1:90 after 200 days. In the purified group A substance, the complete inhibition, which was 1:27000 on the very first day, was reduced to 1:1000 aftir 200 days, and in the group B substance of titer of 1:243-000 on the very first day the inhibition was reduced to 1:3000 after 200 days.

In case of leaving in the open test tubes at the room temperature the group A saliva, whose complete inhibition was 1: 270, and the group B saliva, whose perfect inhibition was 1 : 90 times, completely lost their inhibiting activity after 200 days elapsed.

We collected samples of group B saliva again and left it in summer at room temperature. Some turned yellow and others had a strong putrid smell. And we found that the former which had at first an inhibition titer of 1 : 810 was reduced to 1: 30, and the latter lost the complete inhibition.

Once again we separated the carbohydrate like substance from the saliva above tested having been thought to have lost the complete inhibition, and tested the antiagglutinating power of it finding that there was no complete inhibition when diluted to 1: 1000, while when diluted from 1: 100 to 1: 150, it still showed the complete inhibiting activity. And as to those which were kept in ampulla, group A saliva and its purified group substance, the group B saliva, and its separated group substance still had the complete inhibiting activity even on the 250 th day, but in the 375 th day's test, both the group A saliva and the group B lost the perfect inhibition entirely. The purified group substances still held the inhibiting activity.

According to the strength of the blood cell used for agglutination test there is of course the difference in thə inhibition of agglutination, and through it is not proper to emphasize that the inhibitory force is reduced accurately to so and so times dilutions we are non-the-less sure in our test the inhibition of the purified group substance will still remain here after.

In solution of group substance, it seems that the antiagglutinating activity is reduced according to the Mays of preservation, and when it is preserved in the sterilized ampulla, the perfect inhibition still exists even when it is kept for 200 days, but when kept in the open test tubes the power is entirely lost and the perfect inhibition of strong putrid smell is entirely lost. These above mentioned results may be explained by the so-called bacterial theory held by Witebsky, Satoh and others (*12*). Thus it is very clear that the group specific substance has the inhibiting force for a fairly long time and it seems that the higher the titer of inhibition is and the more complete the exsiccation is, the stabler their inhibiting activity is.

THE INFLUENCE OF BACTERIA

We put platinum earful of *Bacillus coli*, *Staphylococcus*, *Streptococcus*, *Pneumococcus*, and *Pyocyaneus*, cultivated on common agar media for 18 hours, into 1 cc. of sterile solution of 10 times diluted saliva and sterile solution of group substances dissolved to 1 : 1000, left them at room temperature, tested six hours later the rise and fall of the inhibition and found the complete inhibition was preserved. So we left them for 24 hours and again tested the reduction of the inhibition, but it had the same titer with the one of the controls. Before this experiment we tested, as a preliminary experiment, the influence of the bacteria above mentioned upon the agglutinant, and we found *Bacillus colli*, *Streptococcus* and *Pneumococcus* had no snfluence, while *Staphylococcus* and *Pyocyaneous* reduced the pwer both of agglutinants α and β, as far as half, comparing with that of the control. Haraguchi (*13*) let *Staphylococcus albus*, *Streptococcus* and *Pyocyanes* act on α and β respectively and saw that to α, the influence of *Staphylococcus* was strongest and it reduced the specific alutination titer of the corresponding blood cell about till 1/6° *Streptococcus* till 1/8, and *Pyocyanes* till 1/8, severally, and to β, staphylococcus reduced about till 1/8, *Streptococcus* till 1/16, *Pyocyanes* 1/2. From the above mentioned we can say that some kinds of bacteria seem to reduce the agglutination in some degree. But in our experiment it was not suffered very much.

Schiff (*14*) saw in 1939 that A substance was broken by Welchii bacteria, and Nakamura (*15*) in 1943 cultivated anærobia in the Bouillon, adding the human mekomium carbohydrate, to it like substance by Varney's phosphor combustion method, made the bacteria suspension, or its filtrate act on the above mentioned matter, and saw in either case, there was a destructive action on human mekonium group specific substance. So we made the following experiment in order to confirm if anaerobia was concerned with the destruction of the group substance in human saliva.

We first cultivated anærobia at 37° in Liver-Liver-Bouillon for the following hours thought (to be) the most suitable for growth.

Bac. Welchii	24 hours
Vibrion septique	24 hours
Bac. histolyticus	48 hours

And from these cultivates we separated bacteria by centrifugation, washed two or three times with the sterile physiological saline solution and let it act in such a way to examine the influence of the bacteria upon the group substance from saliva and the spittle itself.

We added a platinum earful of the bacteria to 5 ml. of the group A, the group B sterilized saliva of 1 : 10 dilution and the sterile solutions of group A and B substance of 1: 1000 dilution, left them in the incubators at 37° for 24 hours with the control without adding no bacteria to it to make each test of antiagglutinating activity. According to the test, every group substance in human saliva showed the same titer as the control and no destruction of the specific substance could be seen. According to Nakamura's test, these bacterial suspension seems to act on the human mekonium group substance more effectively (though only a very little) than Varney's phosphor combustion method, but on the contrary we could not attain the expected result. In order to see whether our bacteria incuvated for 24 hours, were still living or not, transplanted two platinum earfuls of them in the Liver-Liver-Bouillon, and cultivated for 24 hours in the incubator at 37°, and then we saw excellent gas formation as the sign of their living. We omit here the table of this experiment. As the above mentioned we got the result that the washed anærobia suspension had no influence upon the group substance in human saliva. So we continued further investigations. We added saliva of group A and group B to the following culture media to make 1: 10 dilution and separated group substance to make 1: 1000 dilution. The basal media was 5 ml. of 2% septone polution of pH 7.6 adding 0.1 ml. of 5% vitamine C. To this media with group substance we inoculated a platinum earful of anærobia cultvated in the Liver-Liver-Bouillon; then together with the control we left them in the incubator for 24 hours and tested the agglutination inhibiting reaction.

The peptone use in this culture was Teruchi's peptone (which was) proved to be free from group substance.

Vitamine C had no influence upon the group substance in my test. According to the experimental result, the complete inhibition in group A saliva, which in case of the control was 1 : 270, was reduced to 1 : 30 by the action of *Bac. Welchii*, to 1 : 90 by *Vibrion septique*, to 1: 90 by Bac, histolyticus; in the group B saliva the complete inhibition, Which in case of the control was 1 : 810, was reduced to 1 : 90 b Bac*Bac. Welchii*; 1 : 270 by *Vibrion septique*; 1 : 90 by *Bac. histolyticus.* In group A substance the complete inhibition, which in case of the control was 1 : 27000, was reduced to 1 : 3000 by *Bac. Welchii*; 1 : 3000 by *Vibrion septique*; 1 : 3000 by *Bac. histolyticus.* I group B substance the complete inhibition, which in case of the control was 1 : 81000, was reduced to 1 : 3000 by *Bac. Welchii*; 1 : 9000 by *Vibrion septique*; 1 : 3000 by *Bac. histolyticus.*

As in the above test we experienced that the group substance of human spittle added to the peptone media containing vitamine C was broken by the action of the bacteria. Again we made the following experiment in order to see if the group A substance contained in peptone itself would be broken or not when we used the peptone containing group A substance as culture media. Using 2% sterile solution of Witte peptone added with 0.1 ml. of 5% Vitamine C to 5 ml. of it, making group A substance as a basal medium, and inoculating in it *Bac. Welchii*, *Vibrion septique*, and *Bac. hystoryticus*, we cultivated them with the control in the incubators at 37° for 24 hours and tested the antiagglutination reaction.

According to the result the complete inhibition of the 2% peptone (group A) which in case of the control was 1 : 30, was quite lost in every case. Seeing that the washed bacteria had no influence upon the group substance in human saliva, and that the cultivation on peptone solution added with vitamine C clearly showed the destruction of group substance, we can reason out that the group substance would be decomposed by the nascent state of enzyme on the way of the growth of bacteria.

THE INFLUENCE OF CHEMICALS, SACCHARIDES AND ENZYME

We suspected if there was any influence of solutions of medicine, saccharides, and enzyme upon the group specific inhibition in human saliva. We added the original fluid of group A and group B saliva, and 500 times diluted solution of group substances to the same amount of those solutions to be tested, and let them act together for 5 days. After this, making the saliva ten times diluted, and the separated group substances till 1000 times diluted, are tested the reductidn of the inhibiting activity comparing with the control and got thə following results. We made the chief experiment after seeing in preliminary test if those to be tested will give some influence or not upon the agglutination reaction test.

The Influence of Chemicals—According to Yoshida (*16*), even if one soaks the oral mucous membrane in carbolic acid (0.5%) and formalin (10%) for 30 minutes, the group specifity will not be lost, while acid will be weakend a little, and alkali damaged exceedingly, At this, we tested the following chemicals and tested their influence upon thə inhibiting action. We neutralized acid and alkali after definite time of their action and then let α and β sera work on it. As for acid and alkali, we used hydrochloric acid ($N/10$), caustic soda ($N/10$), and as for disinfectant and oxidizing agents, we used Rivanol (1 : 2000) hydrogen peroxide (1000 times), tincture of iodine (3 times) phenol

(0.5%), formalin (10%), cresol (10%), As for colloidal or polymarecular substances, we used sodium nucleinate (1%), coagrot (1%), goldsol reagent (for the colloidal test of cerebrospinal fluid) and as for the reducing agents, we used vitamine C (5%), sodiumhydrosulfite (1%), cysteine (1%), stannous chloride (1%), sodiumhyposulfite (1%) and hydroquinone (1%).

When we used stannous chloride, the specifity of group A and group B saliva and purified group substances were completely lost while other chemicals had no different effect from that of the control; namely the complete inhibition in group A soliva at 30 times, in group A substances at 27000 times, in group B saliva at 90 times, in its purified group substance at 81000 times dilution.

The Influence of Different Sort off Sugars—As for saccharides, we used glucose (1%) (Takeda), fructose (1%) (Merck), saccharose (1%) (Takeda), mannose (1%) (Merck).

As for the result, we could see no influence at all.

The Influence of Enzymes—As for enzymes, we used protamylase (1%) (Takeda), arlco-urease (1%) (Arlington chemical), lypase (1%) (Osaka Bacterial Research Institute), panktase (1%) (Goto Fu-undo), trypsin (1%) (Merck), papayotinum (1%) (Merck), diastase (1%) (Merck), diastase (1%) (Komet), diastase (1%) (Kashiwagi), pepsin (1%) (Grübler), pepsin (1%) (Merck), pepsin (1%) (Sankyo), *etc.*

As for the result, in all cases they all showed the same inhibition titer with those of the control and we could see no influence at all.

In the preliminary experiment, all the pepsin preparations made by Grübler, Merck and Sankyo showed the specific inhibition of type agglutination. We omitted the table of this experiment.

ON THE SO-CALLED BLOOD GROUP ENZYME

There are two theories on the so-called blood group enzyme, and in the one which Schiff and Akune (*17*) advocate, they say it will be the enzyme like substance secreted from the cell of the body itself, and in the other, which Witebsky and Satoh advocate, they say the group destructive factor is not from the body itself as Schiff and Akune say, but will be concerned with the microbe in the human secretions or evacuations, and will derive from outside of the human body cell (namely they hold the bacteriological theory). And, in 1935, in concideration of the fact that the polysaccharides of *Pneumococcus* III are destroyed by a certain kind of enzyme, Landsteiner (*18*) made Morgan's *Mycobacterium* and *Saccharobacterium ovale* operate on group A substance, and stated that the group substance lost its effect; namely, by Morgan's *Myxobacterium*, the activity was reduced as far as from 2 to 10 per cent, and by *Saccharobacteriu ovale* after 20 days, the activity was reduced to 1 per cent.

On the blood group enzyme, though it has been precisely studied by many specialists from several angles ever since, the nature of group enzyme is not known yet.

In this report, we have already studied on the physical and chemical influences upon the group substance in saliva, and experimenced that the group substance has fairly powerful resistance. Under the supposition that the agglutinant element will be destroyed and lose its activity not only by bacteria but also by animal enzyme, we tested anails, earthworms, the intestines of bees and cattle. First of all, we put these materials into a mortar and ground with physiological saline solution and sands; namely we treated 3.5 g. of nails with 20 ml. physiological saline solution, 5 g. of earthworm with 10 ml. physiological saline solution, 1 g. of bees intestine with 5 ml. of physiological saline solution, 3.5 g. of cattle intestines with 20 ml of physiological saline solution respectively, added toluol to each of them, left them in an ice chamber for a week, clarified though centrifugation and filtration, mixed in the ratis of 0.1 ml. (4), 0.2 ml. (5), 0.3 ml. (6) respectively of the enzyme solution with 0.3 ml. of each of the saliva (10 times) of the solution of the group substance (1000 times) let them act each other, and considering the reduction of the force by putrefication, mixed 0.3 ml. of saliva (10 times), or the group substance (1000 times) with 0.1 ml. (1), 0.2 ml. (2), 0.3 ml. (3) of water as the control, and besides this, we mixed 0.3 ml. of water with 0.1 ml. (7), 0.2 ml. (8), 0.3 ml. (9) of the enzyme each to watch the group destructive action at various intervals. The result was as follows:

In group A saliva (Inhibition 270 times),

When the extract of snails was used, at the set (4) after 100 hours it showed the agglutination (±) after 150-500 hours (+), at (5) after 100 hours (+), after 150-500 hours (⧺), at (6) after 50-100 hours (+), (+), after 150-500 hours (⧺),

and it lost the perfect inhibition. When the extract of earthworms was used, at (4) it showed the complete inhibition, and at (5) after 200-500 hours (+) at (6) after 100-350 hours (+) after 400-500 hours (⧺).

When the extract of cattle intestine and bees were used, the inhibiting force of the saliva was not lost in either case and showed the same complete inhibition as that of the control.

In purified group A substance (inhibition titer 2:20000), when the extract of snails was used, at the set (4) and (5), it showed the same complete inhibition just as (1), (2), and (3), at (6) after from 100 to 500 hours, and showed the agglutination (+), that is, slight influence was seen.

When the extract of earthworms was used, at (4) and (5) it showed the complete inhibition, at (6) after 100 hours it showed (±), after from 190 to 500 hours (+), and the complete inhibition was lost).

When the extract of the bee and cattle's intestines were used, at (4), (5) and (6) it showed the same complete inhibition as the controls (1) (2) (3).

As to group B salivea (Inhibiting titer 1:810) when the snail extract was used, it showed at (4) (+) after 150-400 hours, (⧺) after 450-500 hours, at (5) (+) after 100-300 hours, (⧺) after 350-500 hours, at (6) (+) after 100 hours, (⧺) after 150-400 hours, (⧻) after 450-500 hours, that is, the same agglutination as the controls (7) (8) (9) (no more inhibiting activity).

When the extract of earthworm was used upon the group B saliva, it was shown at (4) complete inhibition, at (5) (+) after 200-500 hours, at (6) (+) after 150-200 hours, (⧺) after 250-500 hours, (no complete inhibition).

The bee and cattle's extract showed at any intervals no effect, (the same complete inhibition as the control).

As to separated B-substance (inhibitory titer 1:243000), when the snail extract was used, it showed at (4) (5) complete inhibition, at (6) (+) after 350-500 hours, and in the case of earthworm, at (4) and (5) the complete inhibition, at (6) after 400 hotrs it showed (±), after from 450 to 500 hours, showed (+), and it seems to have lost slight inhihiting activity.

In the case of the extracts of the intestines of bees and cattle, every one showed the complete inhibition like the controls.

Generally speaking, as above written, if the extract of snails and earthworms act on group A and B saliva, the inhibiting force of it is lost, and if we compare these two, it seems the extract of snails was stronger in destructing power than that of earthworms; and against the separated A and B-type substance, the extract from snails and earthworms showed a weak destructive force.

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ON THE TUBERCULOSTATIC ACTIONS OF VARIOUS ORGANIC COMPOUNDS

III. BENZENE, PYRIDINE AND THIAZOLE DERIVATIVES

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The antitubercular activity *in vitro* of several sulfa compounts was reported in the previous paper (*1*), in which the author noticed the potent action of some thiazole derivatives. Meanwhile it was reported by Friedemann *et al.* (*2*) that 2-butoxy, 5-amino derivatives of pyridine and benzene showed to be highly active *in vitro.* This leads me to test the antitubercular action of more simpler aromatic ring compounds without containing the sulfone or sulfanylamide groups. Such as listed in Table I-IV. The results are reported in this paper.

EXPERIMENTAL

The experimental methods are quite the same as those of the previous report (*1*), except that a compound is considered *inactive* if the growth of bacilli at the surface of the media is not inhibited at the concentration more than 1 : 10,000.

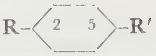
In Table I are listed several benzene derivatives. Here, three butoxy or propyloxy amino-benzene show a definite activity in simple synthetic Lockeman's medium which does not contain serum.

If the nutrient solution contains serum, like the Kirchner's medium, the activity of those three compounds disappears completely. Moreover, a small amount of submerged growth is always observed in the bottom synthetic media even when the surface growth is inhibited. In Table I, it may be clear that the length of R-alkyl chain must have some relation to the antibacterial effect, and it seems to be unfavorable to end R-chain by amino group.

It is possible to prepare more various pyridine derivatives than those of benzene. A number of these pyridine derivatives are listed in Table II.

TABLE I

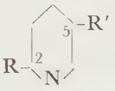
Benzene Derivatives



	R	R′	Min. Concn. at which bacteriostatic action is apparent
1	-butoxy	-amino	1 : 2,560,000
2	-butoxy	-acetylamino	1 : 640,000
3	-propyloxy	-amino	1 : 160,000
4	-propyloxyamino	-amino	inactive
5	-propyloxyacetylamino	-acetylamino	inactive
6	-propyloxyamino	-nitro	inactive

TABLE II

Pyridine Derivatives



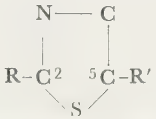
	R	R′	Min. Concn. at which bacteriostatic action is apparent.
7	-oxy	-nitro	inactive
8	-butoxy	-nitro	1 : 10,000
9	-butoxy	-amino	1 : 2,560,000
10	-butoxysulfonyl	-amino	1 : 10,000
11	-butoxysulfonyl	-nitro	1 : 40,000
12	-amino	-nitro	inactive
13	-butylamino	-nitro	inactive
14	-butylamino	-amino	1 : 20,000
15	-mercapto	-nitro	1 : 80,000
16	-3, 3′-dinitrodiphenyl sulfice		1 : 10,000

Again in these compounds, the length of R-alkyl chain may exert influence on the antibacterial activity. In general, amino group attached to the position 5 is more active than nitro group except the compounds 10 and 111. Comparing the compounds 15 and 16, S-S linkage seems to cause less activity than -SH group.

In the previous report (*1*), the author showed that among the heterocyclic sulfacompounds, the thiazole deriavtives are the most promising. But, here the similar derivatives of thiazole and methyl thiazole showed no definite effect against Mycobacterium. The results are indicated in Table III and IV.

TABLE III

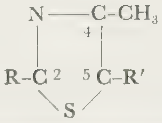
Thiazole Derivaties



	R	R′	Min. Concn. at which bacteriostatic action is apparent.
17	-amino	—	1 : 20,000
18	-butylamino	—	inactive
19	-acetylamino	—	inactive
20	-amino	-chloro	inactive
21	-amino	-nitro	inactive
22	-amino	-bromo	1 : 10,000
23	-acetylbutylamino	-bromo	1 : 20,000
24	-acetylamino	-mercapto	inactive

TABLE IV

Methylthiazole Derivaties



	R	R′	Min. Concn. at which bacteriostatic action is apparent.
25	-amino	—	1 : 20,000
26	-acetylamino	—	inactive
27	-amino	-bromo	1 : 10,000
28	-acetylamino	-bromo	inactive
29	-amilo	-nitro	inactive
30	-amino	-butoxy	1 : 10,000
31	-amino	-butylamino	1 : 10,000
32	-oxy	—	inactive
33	-oxy	-bromo	1 : 20,000
34	-oxy	-nitro	1 : 10,000
35	-chloro	—	inactive

2-Amino thiazole itself is slightly active; but any other substituted groups in the positions 2 and 5 are less active except of 2-acetylamino and 5-bromo thiazole.

2-Amino, 4-methyl thiazole has equal potency to 2-amino thiazole and to 2-oxy, 4-methyl, 5-bromo thiazole. Another derivatives substituted at the position 5 show, in general, weak activities.

DISCUSSION

Among a number of compounds examined, only three ane proved to be highly bacteriostatic *in vitro*; namely, *p*-amino butoxybenzene, *p*-acetylamino butoxybenzene and 5-amino, 2-butoxypyridine. According to Friedemann *et al.* (*2*), the length of alkyl chain must be 4-6 carbon to have maximum antitubercular activity *in vitro* for 5-amino, 2-alkyl oxy pyridine. The postulation may by explained by assuming some physicochemical properties of the compounds in relation to the affinity to the lypophylic tubercle bacilli (*3*). The fact that those three compounds are all inactive in media containing serum supports also this view, because the presence of serum brings about a profound change in the physicochemical properties of the media. Forrest *et al.* (*4*) demonstrated that albumin and relatively small amount of Tween 80 inhibit completely the action of 5-amino, 2-butoxy pyridine. Moreover, Corper *et al.* (*5*) reported recently that their least toxic substance, 2-butoxy, 5-amino pyridine Na formaldehyde bisulfite is ineffective against experimental tuberculosis in guinea pigs, because of the antagonistic action of tissue components. Those disapointing facts lead the author to loose completely interest to work further *in vivo* experiment.

As is well known, niacin is essential for the growth of bacteria, being indispensable in the formation of Co-enzyme I and II, and sulfapyridine or pyridine 3-sulfonic acid is proved to be the antagonist to the Co-enzyme (*6*, *7*), the antibacterial action of the compounds in Table II might be the results of such an antivitaminic action. But, curiously enough, nicotinic acid amide itself shows very strong antitubercular action *in vivo* (*8*). Lwoff (*9*) suggested that certain bacteria used nicotinic acid not only as a component of Co-enzyme I, but also as hematin which turn out later to hemochromogen. From this view, the antibacterial action of pyridine derivatives would not be considered only as the antagonist of Co-enzyme I and II. The structure of those pyridines derivatives in Table II are rather similar to pyridoxine and its related compounds.

About the derivatives of thiazole and 4-methyl thiazole, the antibacterial effect seems to approach more closely to the data given in the series of substituted thiamin components by Schopfer (*10*), Robbins and Kavanagh (*11*), Bonner and Erickson (*12*). According to their work, 2-amino substituted thiazole is fairly inactive to the growth of Staphylococci and also 5-methyl, ethylor hydrogen substituted thiazole showed the same ineffective results for their growth. In the Table IV and V, 2-amino thiazole groups including 5-butoxy, 2-amino thiazole are toxic to the growth of tubercle bacilli. It is interesting to suppose that these antibacterial thiazole derivatives act on the *Mycobacterium* as an potent antivitamin because thiamin can stimulates to grow certain species of *Mycobacteria* (*13*).

SUMMARY

The author tested thirty five compounds of benzene, pyridine and thiazole derivatives against *Mycobact. tubercurosis var. Gallinaceum.* Among these, only 2-butoxy, 5-amino pyridine or benzene derivatives are highly active in synthetic medium, but inactive in Kirchner's Medium. The mode of action of some compounds are discussed.

The author is most grateful to Prof. K. Kodama and Prof. M. Ishidate for their helpful advices and especially to Dr. Y. Yamamoto who gave generously the almost all compounds used in the study of this series.

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The Journal of Biochemistry, Vol. 38, No. 1.

STUDIES ON THE GLUCOSE DEHYDROGENASE OF ASPERGILLUS ORYZAE

By YASUYUKI OGURA

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It has been shown in previous works (*1, 2, 3*) that the glucose dehydrogenase extracted from mycelium of Aspergillug oryzae displays a number of features which distinguish it from similar enzymes reported earlier by other workers (*4, 5, 6, 7*). In the present paper further characteristics of the enzyme were studied, particularly with an eye toward the problem of the possible significance of phosphate in the enzyme reaction and the specificity of the enzyme in its mutual action with various redox-dyes.

METHODS

Preparation of Enzyme—The enzyme material was prepared essentially as described in previous report (*3*). The finely triturated mycelium of *Aspergillus oryzae* was extracted for 20 hours with 0.5 *M* phosphate buffer (pH 8.2). The extract was dialyzed 3 hours through collodium membrane against water, 5 volumes of ice-cold acetone was added at 0°, and the resulting precipitate was redissolved in water, and dialyzed. To this solution, saturated solution of lead sabacetate was added until no more precipitate occurred, the precipitates discarded by centrifugation and the excess of lead in the supernatant was removed by addition of 0.5 *M* phosphate buffer (pH 7.2) followed by dialysis against water. To the solution, 6 volumes of ice-cold acetone was added at 0°. The precipitate was centrifuged, and dried in a vacuum dessicater. The yellowish powder thus obtained was used as the enzyme preparatioh. Test with various substances showed that this preparatson contains no dehydrogenases other than glucose dehydrogenase.

Measurement of the Reaction and the Redox-potential—The rate of reduction of redox-indicators was measured by using Thunberg's tubes; enzyme-buffer solution placed in the main space and redox-indicotor and substrate solution in the side bulb. After the attainment of thermal equilibrium at 30°, both solutions were mixed and the time of reduction was measured. The measurement of the redox-potential of reaction mixture was effected by using the Thunberg-Borsook tube, the main space, provided with platinum electrode, was filled with the enzyme solution and the side bulb with a solution of redox-dyes, D-glucose and D-gluconate. After the vessel was evacuated and filled with N_2-gas, the solution in the side bulb was dumped into the main chamber and the course of potential change was followed electrometrically. (Temp.: 15°.)

RESULTS

Oxidation of D-*Glucose in the Presence and Absence of Phosphate*—In order to determine whether D-glucose is oxidised to gluconic acid directly or through phosphorolysis, the reduction velocity of thionine in the presence and absence of phosphate was compared. Using phosphate buffer on the one hand, and veronal buffer on the other (in both cases pH was 7.2 and the final concentration of buffers, 0.05 M) it was found that the reduction velocity of thionine was quite independent of the existence of phosphate.

*Oxidation of 6-Phospho-*D-*Glucose*—According to Warburg, *et al.* (*9*), Robison-ester dehydrogenase oxidises 6-phospho-D-glucose to 6-phospho-D-gluconate in the presence of co-enzyme II. It was stated by Harrison (*6*, *7*) and Das (*8*), on the other hand, that the glucose dehydrogenase from liver oxidises not only D-glucose in the presence of co-enzyme I or II, but also 6-phospho-d-glucose. It is of interest to study whether 6-phospho-D-glucose will be oxidized by the enzyme of *Aspergillus oryzae*.

TABLE I

The Oxidation of D-Glucose and 6-Phospho-D-Glucose

0. 5 ml., buffered enzyme solution (1 mg. enzyme preparation per 0. 5 ml.); 0. 5 ml. redox-indicator (1 : 5000); 1. 0 ml., 0. 1 M substrate solution; pH 7. 2; temperature, 30°.

Substrate	Decolorization time (min.)	
Redox-indicator	D-Glucose 0. 05 M in the final concentration	D-Glucose-6-phosphate 0. 05 M in the final concentration.
Thionine.	9	150
2, 6-Dichlorophenol-indo-phenol.	5	120

As may be seen from Table I, the dehydrogenation of 6-phospho-D-glucose occurred much more slowly than that of glucose. From this and the foregoing results, it may be concluded that our enzyme oxdizes D-glucose directly without the participation of phosphate.

Effect of Gluconate on the Oxidation Rate of Glucose—The reduction time of thionine in the presence of 0.02 *M* glucose and 0.2 *M* gluconate in final concentration was compared with the one in the presence of 0.02 M glucose only. In both cases the reduction velocities of thionine were found to be entirely the same. After thionine was completely reduced in the presence of 0.02 *M* D-glucose, 0.2 *M* gluconate in the side bulb was poured into the main chamber, whereby no sigh of reoxidation of the leuco-dyestuff was observed. These observations indicate that the oxidation by our enzyme of glucose to gluconate proceeds irreversibly.

Change of Redox-Potential in the Reaction System—Irreversibility of the reaction in question also manifests itself in the course of change in the redox-potential of the reaction mixture. In Fig. 1 are shown the results obtained when she following redox-doyes were added to the reaction mixture: thionine, cresyl blue, methylene blue, indigotetrasulfonate, capri blue, indigotrisulfonate, indigodisulfonate, nile blue, cresyl violet, brilliant alizarine blue, phenosafranine, Janus green and neutral red. As previously reported, of these dystuffs only thionine can be rapidly reduced by our enzyme while others are almost or entirely not reduced. The values of normal redox-potential E_0' of these dyestuffs are ranged successively from 0.062 volts to −0.340 volts at pH 7.0, with the average difference of about 0.040 volts. Composition of the reaction system was as follows: enzyme solution (1 mg. enzyme preparation per 0.5 ml.) 0.5 ml.; 0.5 *M* phosphate buffer, 0.5 ml.; D-glucose solution,

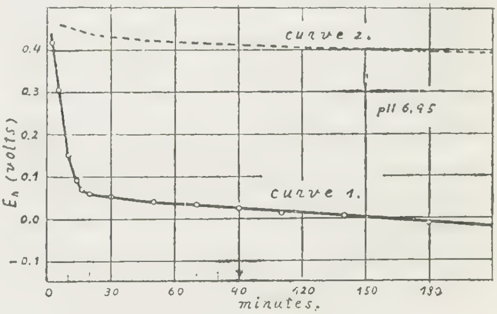

FIG. 1. The course of potential change in the reaction systen with thionine and a number of other days as redox-indicators.

Curve 1: With D-glucose (final concentration 0.1 *M*) in the presence of varying concentrations of D-gluconate (1.0, 0.5, 0.1, 0.01. and 0 *M*)

Curve 2: Without the addition of D-glucose in the presence and absence of 0.1 *M*. D-gluconate.

1.0 ml.; D-gluconate solution, 2.0 ml. and each of the redox-dyes (1 : 5,000), 0.3 ml. The total volume of reaction mixture was made up to 10.0 ml. with distilled water. The concentration of gluconate was varied between 0 and 1.0 *M*, so as to make the ratio of [glucose]/[gluconate], 1 : 10, 1 : 5, 1 : 1, 1 : 0.1, and 1 : 0, respectively.

It was found that the difference in the ratio of [glucose]/[gluconate] did not cause any difference in time course of the redox-potential which in all cases decreased quite rapidly within 15 minutes, and after bending abruptly at $E_h = +0.062$ volts, the curve went down very gradually, but steadily, attaining no final value even after 54 hours. In the absence of glucose, the E_h-value remained high, showing only a slight decrease with the lapse of time.

It should be noted that the time course of the potential change in the presence of glucose is somewhat different according to the pH of the medium. In Fig. 2 are given the curves obtained at pH 6.2, 7.2 and 8.2, respectively. The E_h-value at which the curve shows an abrupt bending was 0.090 volts at pH 6.2, 0.055 volts at pH 7.2 and 0.025 volts at pH 8.2. These values coincide quite well with the E_0'-values of thionine (0.088 volts at pH 6.2, 0.056 volts at pH 7.2 and 0.025 volts at pH 8.2) which, among the dyestuffs used in the experiments, represents the most suitable hydrogen-acceptor for the action of oure enzyme.

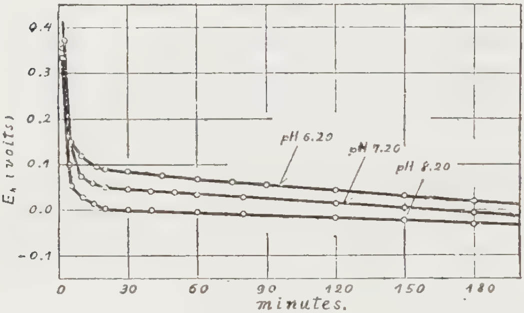

FIG. 2. The effect of pH on the course of potential change in the reaction system with thionine and a number of other days as redox-indicators. The final concentration of D-glucose, 0.1 *M*. At each pH-value the same curve was observed when D-gluconate was added in the concentration of 0.5, 0.1, or 0 *M*, respectively.

The time cours of the change in E_h is quite different according as the sort of redox-dyes added to the reaction mavture. In Fig. 3 are shown the curves obtained when a mixture of the following dyes was added: *o*-cresol-indo-2, 6-dichlorophenol, toluylene blue, cresyl blue and methylene blue. Of these, *o*-cresol-indo 2, 6-dichlorophenol reacts by far the most effectively with the enzyme. In this case also, the E_h-

value (0.170 volts) at which the curve bended abruptly coincised practically with the E_0'-value of the most suitable dyestuff.

Fig. 4 shows the results obtained when the mixture of the following redox-dyes were added: methylene blue, capri blue, indigotetrasulfonate, indigotrisulfonate, indigodisulfonate, nile blue brilliant alizarine blue, phenosafranine, Janus green and neutral red. All these substances are "unsuitable" hydrogen-acceptors for our enzyme, but among them, methylene blue is relatively the best. In this case, the lowering of the E_h''-curve occurred very slowly, but a distinct bending was observed at about 0.00 volt which is practically equal to the E_0'-value (0.0075 volts) of methylene blue at the pH (7.08) of the experiment.

Acceleration of the Reduction of "Unsuitable" Redox-Dyes in the Presence of "Suitable" Ones—In the course of the above experiments it was

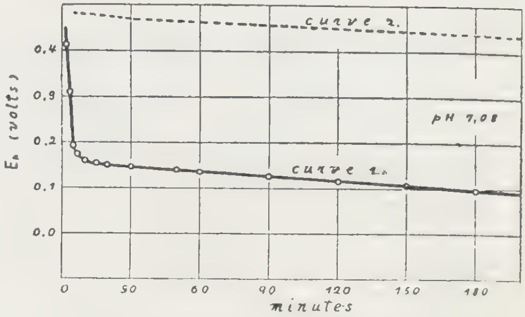

FIG. 3. The course of potential change in the reaction system with *o*-cresol-indo-2, 6-dichlorophenol, toluylene blue, cresyl blue and methylene blue as redox-indicators.

Curve 1: With D-glucose (final concentration, 0.1 *M*) in the presence of varying concentrations of D-gluconate (0.5, 0.1, and 0 *M*).

Curve 2: Without the addition of D-glucose.

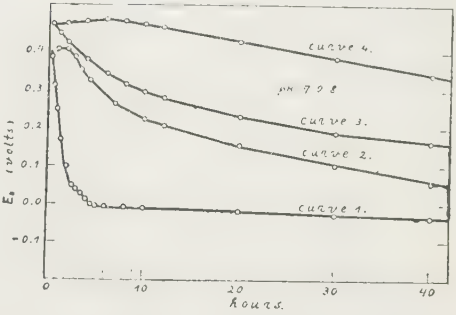

FIG. 4. The course of potential change in the reaction system with methylene blue and some other unsuitable days as redox-indicators.

Curve 1: With D-glucose (final concentration: 0.1 *M*) in the presence and absence of D-gluconate (0.5 *M*).

Curve 2: Without the addition of D-glucose.

Curve 3: With D-glucose but without the addition of redox-indicators.

Curve 4: With D-glucose and redox-indicators but without the addition of the enzyme.

TABLE II *Effect of Thionine on the Rate*

0. 5 ml., buffered enzyme solution (ph 7. 2 and 1 ml. enzyme preparation
0. 5 ml., D-glucose solution (0. 2 M). Total volume was made up to 2. 0 ml. with

Redox-indicator	Thionine	Cresyl blue			Methylene blue			Capri blue			Indigotri-sulfonate		
The value of E_0' at pH 7. 2	+0. 056 volts	+0. 027 volts			+0. 004 volts			−0. 007 volts			−0. 091 volts		
Thionine (ml.)	0. 5	0. 5	—	—	0. 5	—	—	0. 5	—	—	0. 5	—	-
Redox-indicator (ml.)	—	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5
D-Glucose (0. 2 M) (ml.)	0. 5	0. 5	0. 5	—	0. 5	0. 5	—	0. 5	0. 5	—	0. 5	0. 5	—
Time of decolorization (minutes)	12	40	55	>300	52	150	>300	71	240	>300	250	300	>300

found that those dyestuffs, which are by themselve incapable or almost incapable of functioning as a hydrogen-acceptor for our enzyme, are more or less rapidly reduced when a suitable dyestuff such as thionine or *o*-cresol-indo-2, 6-dichlorophenol was present. To investigate this phenomenon more in detail, the decolorization time of various dye-stuffs in the presence and absence of suitable dyes was compared.

In the experiments given in Table II, thionine was used as the suitable dye. Of interest is the fact that the acceleration by the presence of thionine was observed with cresyl blue, methylene blue, capri blue

TABLE III

Effects of Suitable Redox-indicators on the Reduction Rate of Toluylene Blue

0. 5 ml., buffered enzyme solution (ph 7. 2 ana 1 mg. enzyme preparation per 0. 5 ml.); 0. 5 ml., *o*-cresol-indo-2, 6-dichlorophenol solution ($6.0 \times 10^{-4} M$), or *p*-quinone solution ($10^{-3 \cdot 0} M$); 0. 5 ml., toluylene blue solution ($6.0 \times 10^{-4 \cdot 0} M$); 0. 5 ml., D-glucose solution (0. 2 M). In all cases, the total volume of reaction medium was made up to 2. 0 ml. with redistilled water. Temp., 30°.

Redox-indicator and substrate	The value of E_0' at pH 7. 2	A	B	C	D	E (ml.)
o-Cresol-indo-2, 6-dichlorophendl	+0. 166 vOlts	0. 5	—	—	—	0. 5
p-Quinone	+0. 257 volts	—	—	0. 5	—	—
Toluylene blue	+0. 108 volts	0. 5	0. 5	0. 5	0. 5	—
D-glucose (0. 2 M)		0. 5	0. 5	0. 5	—	0. 5
Time of decolorization (minutes)		21	100	100	>300	10

of Reductiou of Unsuitable Redox-dyer

per 0. 5 ml.); 0. 5 ml., redox-indicator ($6\times10^{-4}\,M$); 0. 5.ml, thionine ($6\times10^{-4}\,M$); redistilled water. Temp., 30°.

Indigodi sufonate			Nile blue			Brilliant arizarine blue			Phenosafranine			Janus green			Neutral red		
−0. 134 volts			−0. 150 volts			−0. 185 volts			−0. 250 volts			−0. 270 volts			−0. 351 volts		
0. 5	—	—	0. 5	—	—	0. 5	—	—	0. 5	—	—	0. 5	—	—	0. 5	—	—
0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5	0. 5
0. 5	0. 5	—	0. 5	0. 5	—	0. 5	0. 5	—	0. 5	0. 5	—	0. 5	0. 5	—	0. 5	0. 5	—
>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300

and indigotrisulfonate, but not with indigotrisulfonate, nile blue, brilliant alizarine blue, phenosafranine, Janus gerrn and neutral red. Obviously the capacity of thionine in accelerating the decoloration of each unsuitable dye is dependent on the E_0'-value of the latter, and indeed, for the acceleration to take place, it cannot differ from that of thionine by more than 0.140 volts which was the case with indigotrisulfonate.

When *o*-cresol-indo-2, 6-dichlorophenol was used as suitable dyestuff, accelerated reduction was observed with toluylene blue but not with cresyl blue or methylene blue. On the otner hand, *p*-puinone, though a suitable dyestuff by itself, exerts no influence upon the reduction of toluylene blue, cresyl blue and methylene blue. For *o*-cresol-indo-2, 6-dichlorophenol and *p*-quinone the necessary condition for ac-

TABLE IV

Reduction of Methylene Blue Accelerated by the Addition of Thionine in Various Concentrations

0. 5 ml., buffered enzyme solution (1 mg. enzyme preparation per 0. 5 ml.); 0. 5 ml., methylene blue solution ($6.0\times10^{-4\cdot0}\,M$); 0. 5 ml., thionine solution; 0. 5 ml., D-glucose solution ($0.2\,M$). Temp. 30°.

Redox-indicator	A	B	C	D	E
Thionine ia final concentration (M)	$1.5\times10^{-4\cdot0}$	$0.75\times10^{-4\cdot0}$	$0.37\times10^{-4\cdot0}$	$0.2\times10^{-4\cdot0}$	0
Methylene blue in final concentration (M)	$1.5\times10^{-4\cdot0}$	$1.5\times10^{-4\cdot0}$	$1.5\times10^{-4\cdot0}$	$1.5\times10^{-4\cdot0}$	$1.5\times10^{-4\cdot0}$
Time of decolorization (minutes)	45	53	70	85	125

celerating the reduction of other unsuitable dyestuffs seems to be that their E_0'-values do not differ by more than 0.060 volts.

DISCUSSION

Of interest is the fact that the reaction catalized by our enzyme shows a rather marked specificity towards hydrogen acceptors, preferring, on the whole, the dyestuffs with higher E_0'-values such as thionine, *o*-cresol-indo-2, 6-dichlorophenol, Bindschedler-green and *p*-quinone. That, however, the E_0'-value is not the only determining factor for the applicability of dyestuffs as hydrogen acceptors may be seen in the case of toluylene blue which, though with the E_0'-value of as high as $+0.115$ volts (pH 7), could not be reduced unless some suitable dyes were added.

Since the reaction glucose→gluconic acid $+2H^+ + 2e$ is an irreversible process, the system must, from a purely thermodynamic standpoint, be regarded as being able to reduce even the dyestuffs with lower E_0'-values. It should be recalled that the Shardinger's enzyme which catalyzes a similar irreversible reaction: aldehyde→acid $+2H^+ + 2e$, can untilize even neutral red ($E_0' = -0.340$ at pH 7) as hydrogen acceptor (*10*). The preference of certain redox-dyes by our enzyme is obviously a matter of purely kinetic significance, rather than a thermodynamic one.

Acceleration of the reduction of unsuitable dyes by the presence of suitable dyes is also to be explained kinetically as being due to the hydrogen-transferring action of the suitable dyes. While the reaction between the suitable dyes and the system glucose→gluconic acid $+2H^+ + 2e$ is irreversible and enzymatic, the reaction between the suitable dyes and unsuitable ones is reversible and non-enzymatic. The fact that the accelerating action in question can be observed only when the difference in E_0'-values of the suitable and unsuitable dyes lie within a certain limit, can also be explained in kinetic terms with the consideration that the efficacy of redox systems acting as intermediary hydrogen-carrier must be largely dependent on the ratio of its oxidation and reduction forms established during the course of the reaction.

SUMMARY

1. The glucose dehydrogenase extracted from *Aspergillus oryzae* oxidizes D-glucose to D-gluconate in the presence of a suitable

hydrogen acceptor. This reaction can take place in the absence of phosphate. On the other hand, 6-phospho-D-glucose was oxidized much more slowly than D-glucose. From these facts it was concluded that the enzyme oxidizes D-glucose directly without the participation of phosphate.

2. The rate of reduction of thionine in the presence of glucose was not modified by the addition of gluconate. Nor did the addition of gluconate cause any sign of reoxidation of thionine which had been totally reduced by the oxidation of glucose through the enzyme. The enzymatic oxidation of glucose to gluconate may, therefore, be regarded as being practically an irreversiblə process.

3. The change of redox-potential in the reaction system (in the presence of various redox-dyes) was followed potentiometrically. It was found that the existence of gluconate in the reaction medium did not exert any influence upon the time course of the potential change. In all cases the potential decreased more or less rapidly up to a certain value and from that point it continued to drop quite slowly but steadily without attaining any final value even after fifty hours. The bending point of the potential-time curve was found to correspond fairly well with the E_0'-value of the dyestuff which was most readily reduced among the dyestuffs used.

4. The reduction of those dyestuffs which by themselves are only slowly or practicallp not reduced by the enzyme system can be accelerated by the addition of easily reducible redox-dyes. For this accelerating effect, however, the necessary condition is that the difference in E_0'-values of suitable and unsuitable dyes should not surpass a certain limit.

(For example the limit is $\varDelta E_0'=0.140$ volts when thionine was used as suitable hydrogen acceptor.) It was briefly discussed that all the facts pertaining to the mutual action between the enzyme system in question and the various redox-dyes studied are to be interpreted from kinetic rather than from a thermodynamic standpoint.

In conclusion, I wish to express my thanks to Prof. H. Tamiya for his constant encouragement and suggestions during the course of this research.

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AMINO ACIDS OF MYCOBACTERIUM TUBERCULOSIS

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INTRODUCTION

The current knowledge of the proteins contained in *Mycobacterium tuberculosis* seems quite incomplete, although extensive investigations have been made of the lipid and carbohydrate components. Workers in this field have already determined the basic amino acids in the hydrolysate. Furthermore, Tamura (*1*) described phenylalanine, valine, and proline; Johnson (*2*) *et al.*, cystine; and Campbell (*3*), tyrosine and glutamic acid. The presence of tryptophan was confirmed by Campbell (*3*) Johnson (*2*), *et al.*

The present investigation was undertaken to determine as many kinds of amino acids in the whole proteins as possible, including the essential amino acids reported by Rose. The sum of nitrogen found in the amino acids was 93.10% of the total nitrogen. The presence of a large amount of several essential amino acids, *e.g.*, methionine, leucine, valine, lysine, and threonine is noteworthly in connection with the treatment of tuberculous diseases.

EXPERIMENTAL

Material—*Mycobacterium tuberculosis* (human strain) was cultivated in Long's non-protein medium (*4*) for 1-1.5 months, filtered by suction, washed with alcohol and ether successively, and dried in a vacuum desiccator.

Removal of Lipids—760 g. of the material was bniled with absolute ethanol for 1 hour and filtered by suction. Aftea repeating this process 5 times, the alcohol was evaporated on a water bath and kept in a vacuum desiccator with solid paraffin. After several days, the mass of bacilli was extracted with ether in a Soxhlet apparatus for more than 120 hours. The ether was removed by evaporation, and the residue boiled for 24 hours with chloroform, and then filtered by suction and the chloroform was evaporated. The mass was extracted again with ether in a Soxhlet apparatus consecutively for 50 hours and dried in a vacuum desiccator. Approximately 356.7 g. of a nearly while, powdery substance was obtained containing 10.06% N, 0.77% P, and 2.5% ash.

Quantitative Determination of Amino Acids—The following amino acids were measured and the results were given in Table I obtained.

TABLE I

Analysis of Amino Acids in Tuberculosis

Amino acids	Methods of determination	Quantity in defatted bacili (%)	Amino acid-N/total-N (%)
Threonine	Shinn and Nicolet (*5*)	3.07	3.59
Methionine	Kassel and Brand (*6*)	7.09	6.62
Cystine	Folin and Looney (*7*)	1.38	1.60
Phenylalanine	Kolleman (*8*)	3.68	3.11
Tyrosine	Folin and Looney (*7*)	1.11	0.85
Tryptophan	Folin and Looney (*7*)	1.59	2.17
Arginine	Van Slyke (*9*)		
Histidene	Kossel and Gross (*10*)	4.63	14.81
	Van Slyke (*9*)		
	Sera (*11*)	1.61	4.33
Lysine	Van Slyke (*9*)	3.86	7.35
Serine	Kuroda and Hirohata (*12*)	2.83	3.75
Ammonia	Van Slyke (*9*)	—	4.87
Humin	Van Slyke (*9*)	—	4.04

Determination of Glycine,aValine, Leucine, Procine, Aspartic, and Glutamic Acids—Schryver (*12*)-Brazier (*13*)'s copper method was used to isolated and determine these amino acids. 103.944 g. of lipid-free bacilli were boiled in 750 ml. of 20% hydrochloric acid for 30 hours, filtered by suction through a sintered glass filter and washed repeatedly with hot water until the washings became colorless. The residue, insoluble in hot hydrochloric acid, contained 0.38% N. The filtrate was concentrated by distillation in *vaccuo*, and all hydrochloric acid removed by repeated evaporation under reduced pressure, adding to it a small quantity of water from time to time. The viscous residue was dissolved in water and diluted to 500 ml. With an aliquot portion of the solution, the total nitrogen was determined and calculated as 9.68% of the dried powder.

The quantity of ammonia in the hydrolysate was estimated by distillation in $N/10$ sulfuric acid under the alkaline reaction with calcium oxide. The basic amino acids in the hydrolysate were precipitated with calcium oxide. The basic amino acids in the hydrolysate were precipitated with phosphotungstic acid and the excess of it in the filtrate removed with amyl alcohol-ether.

Amino acids in the solution were converted into their copper salts by boiling with copper carbonate. The solution was evaporated under diminished pressure and completely dried with absolute acetone. The residue of the copper salts was fractionated by dissolving it in ethanol, methanol, and water. Brazier's method (*14*) was used to separate each amino acid. The results obtained are shown in Table II.

TABLE II

Amino Acid Isolated by Brazier's Method

Amino acids	Quantity in defatted bacilli (%)	Amino acid-N/total-N (%)
Glycine	2. 20	4. 08
Alanine	7. 75	12. 11
Valine	4. 03	4. 79
Leucine	6. 64	7. 05
Aspartic acid	0. 94	0. 98
Glutamic acid	4. 64	4. 38
Proline	2. 18	2. 64

TABLE III

Analysis

Amino acids	Nitrogen content		Melting point
	Calculated	Found	
Glycine	18. 67	18. 10	210°~215°
Alanine	15. 73	15. 55	250°~270°
Valine	11. 96	11. 82	
Leucine	10. 69	10. 45	285°
Aspartic acid	10. 53	10. 26	240°~249°
Glutamic acid	9. 50	9. 45	195°
Proline (Cu salts)*	9. 60	9. 58	

* Cu content of prolin :Cu : Calculated : 21. 78% Found : 22. 04%

The presence of all amino acids mentioned in Tabls I and II, including α-amino-butyric acid, was confirmed by paper chromatography.

The results of N analysis and the melting point of each amino acid are shown in Table III.

In Table IV. The results obtained in this investigation are compared with those of Tamura (*1*), Campbell (*2*) and Johnson *et al.* (*3*)

TABLE IV

Amino Acid-N : Total N% in Defatted Bacilli

Amino acids	Tamura (*1*)	Johnson-Brown (*2*)	Campbell (*3*)	Eguchi	
				Amino acid-N %	Amino acid in proteins %*
Glycine	—	—	—	4. 08	3. 50
Alanine	—	—	—	12. 11	12. 32
Valine	11. 50	—	—	4. 79	6. 41
Leucine	—	—	—	7. 05	10. 57
Serine	—	—	—	3. 75	4. 50
Threonine	—	—	—	3. 59	4. 88
Cystine	—	1. 26	—	1. 60	2. 19
Methionine	—	—	—	6. 61	11. 27
Phenylalanine	7. 31	—	—	3. 10	5. 85
Tyrosine	—	+	0. 73	0. 85	1. 76
Tryptophan	—	+	+	2. 17	2. 53
Proline	3. 09	—	—	2. 64	3. 47
Histidine	1. 53	11. 48	3. 41	4. 33	2. 56
Lysine	1. 49	3. 69	1. 71	73. 5	61. 4
Arginine	7. 64	10. 63	28. 87	14. 81	7. 36
Aspartic acid	—	—	—	0. 98	1. 49
Glutamic acid	—	—	3. 05	4. 38	7. 38
α-Amino butyric acid	—	—	—	+	+
Ammonia	1. 15	—	—	4. 87	
Humin	15. 79	4. 11	9. 13	4. 04	
Sum	49. 50	31. 17	46. 90	93. 10	94. 17

* The percentage of each amino acid was obtained by calculating from its N, assuming that the whole proteins of bacilli contain an average of 16% N.

SUMMARY

From the hydrolysate of the whole proteins of *Mycobacterium tuberculosis*, (human strain) the following 17 amino acids were isolated or estimated: glycine, alanine, valine, leucine, serine, threonine, cystine, methionine, phenylalanine, tyrosine, tryptophan, proline, histidine, lysine, arginine, aspartic acid, and glutamic acid.

The presence of α-amino butyric acid was confirmed by paper chromatography.

The presence of a relatively large quantity of methionine, leucine, valine, lysine, and threonine, each an indispensable amino acid for human nutrition, is worthy of notice.

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NOTE TO THE COLORIMETRIC DETERMINATION OF ARGININE

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In this Journal Vol. 37, page 231, I have reported a new colorimetric method for the determination of arginine. The essential point of this method lines in the development of brown yellow colour of arginine with oxine and hypobromite in alkaline medium. At that time the author recommended that the hypobromite solution is to be prepared by dissolving 2 g. of bromine in 100 ml. of 5 % caustic soda. It was found later that this solution is so strong, that it is better to dissolve 1 g. of bromine. Usually 2-3 drops of this hypobromite solution are enough for the standard arginine solution and 3-6 drops for protein hydrolysate.

In winter a little excess of hypobromite has almost no appreciable effect on the determination, while in summer this is not the case, so that special care should be taken for the use of proper amount of hypobromite. In summer the temperature of the solution under determination rises in the course of the colour comparison, but the determination is not impossible, if the control-test is carried out with the standard arginine solution at the same time.

The blank-test, which is carried out with the reagents in the same quantities except that no arginine is added, remains colourless even after standing at over 20°. If the reagents, however, contains a trace of iron at the concentration of 1/20000 M gives colour distinctly after standing for 30 min. at 5°. But, as the protein hydrolysates are usually diluted over 1 : 20000, it may be said that this interference is generally neglected for the practical use. Needless to say, this determination must be done at low temperature and without delay. Pulfurich's photometer is employed for colour measurements, using the filter S. 50 and putting distilled water as a reference liquid.